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14. ABSTRACT

Telomerase, a ribonucleoprotein enzyme minimally composed of an RNA template (hTR) and a catalytically active protein subunit (hTERT), synthesizes telomeric repeats onto chromosome ends and is obligatory for continuous tumor cell proliferation, as well as malignant progression of breast cancer cells. Telomerase is an attractive anti-cancer therapeutic target because its activity is present in over 90% of human cancers, including more than 95% of breast carcinomas, but undetectable in most somatic cells. Traditional chemo- and radio-therapies lack the ability to effectively control and cure breast cancer, in part because residual cells are or become resistant to DNA damaging modalities.

While various telomerase inhibition strategies cause cancer cells to undergo apoptosis or senescence, there is often a lag period between administration and biologic effect (Corey, 2002). Our goal in this study was to compare the efficacy of different telomerase inhibition strategies in concert with standard chemotherapeutic agents at triggering senescence and/or apoptosis in cultures of breast cancer cells. We hypothesized that telomerase inhibition strategies will sensitize breast cancer cells to traditional chemotherapies, potentially reducing the lag phase, allowing for more potent anti-tumor effects at lower doses, and therefore ultimately imparting less toxicity to the patient.

15. SUBJECT TERMS
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Table of Contents

Introduction

Last year over 211,000 new cases of invasive breast cancer occurred among women in the United States (American Cancer Society, 2005). Currently, the primary treatment for breast cancer consists of surgery and adjuvant therapies including chemotherapy, hormone therapy, and localized radiation. Despite the initial success of these clinical approaches, the frequent recurrence of breast cancer indicates that resistance to therapy is common in breast tumors. Associated with nearly 90% of malignant breast cancer, telomerase is a reverse transcriptase containing a catalytic protein component, hTERT, and an RNA template, hTR, for catalyzing the addition of telomeric (TTAGGG) DNA repeats onto the chromosome ends (Feng et al. 1995; Weinrich et al. 1997). As such a prominent molecular marker for human cancer, telomerase has proven useful for detection of recurrent disease, as well as a promising target for adjuvant cancer therapy, especially for breast cancer treatment (Nakamura et al. 2002). Traditional therapies (surgery, chemotherapy, radiotherapy, etc.) lack the ability to effectively control and cure breast cancer, primarily because residual cells are or become resistant to DNA damaging modalities including standard chemo- and radiotherapies. Since telomerase requires its associated hTR for repeat synthesis, we have chosen to use RNA interference as a method to inactivate hTR and hence telomerase. The siRNAs we will use are directed at the hTR portion of telomerase, which is a modification of the traditional RNAi in that hTR is a functional RNA and not an mRNA RNA interference (RNAi) has become a powerful tool for the analysis of gene function in that RNAi allows sequence specific inhibition of gene expression (Berns, et al. 2004; Elbashir, et al. 2001; Fire, et al. 1998; Hammond, et al. 1998; Li, et al. 2004; Martinez, et al. 2002; McManus and Sharp, 2002; Mourelatos, et al. 2002).

Furthermore, we have elected to examine the effects of inhibiting telomerase by blocking hTERT, using a dominant negative (DN) and/or siRNA, and determining the differences in induction of breast cancer cell sensitivity to standard breast cancer therapies. We have found decreased telomerase activity using both hTR siRNA and DN-hTERT in MCF7 breast tumor cells. Preliminary experiments have shown decreased telomerase activity using the telomere repeat amplification protocol (TRAP), in certain clones of MCF7 cells containing hTR siRNA as well as DN-hTERT cell lines as compared with controls. Over time we have observed telomere shortening, senescence and an increase in sensitivity to Adriamycin (AdR) at lower levels than standard treatments in our DN-hTERT clones, suggesting that the telomeres are more vulnerable to AdR. The increased susceptibility of the cells to DNA damage will be critically important in the induction of apoptosis or senescence, and blocking telomerase will likely prevent proliferative recovery in both sets of cell lines. The experiments here should provide new perspectives on RNAi and clarifications of the cellular response of breast tumor cells to treatment after sensitization by telomerase inhibition, which will be critically important for the identification of adjuvant therapies directed at telomerase for breast cancer patients.

Body

Genetic Inhibition of hTR and Sensitization of MCF-7 Cells

Objective #1: **Maintain stable suppression of hTR using RNA interference with high levels of telomerase inhibition.**

Telomerase is expressed in the majority of human cancers, approximately 90%, as well as being highly elevated in over 70% of immortalized cell lines, thus making its detection extremely promising for aiding in cancer diagnosis and prognosis, as well as viable therapeutic target. (Kim et al. 1994; Shay and Bacchetti, 1997). However, in normal adult somatic cells telomerase is not active, but the RNA component hTR is ubiquitously expressed. Investigators have shown that the full gene dosage of hTR is highly important within the cell, as seen in patients with the autosomal dominant form of non-X-linked dyskeratosis congentia, which in one family pedigree, was caused by haploinsufficiency or one nonfunctional copy of the hTR gene. These patients have progressive bone marrow failure and die in early adulthood or middle age (Vulliamy et at. 2001; Comolli et at. 2002). This suggests that inhibition of telomerase by reducing hTR levels may have deleterious effects on cancer cells.

Attempts to inhibit telomerase activity usually result in gradual telomere shortening followed by a lag period, ultimately resulting in cellular death or senescence. Since the telomerase holoenzyme is composed of multiple components, there are many potential targets for achieving telomerase inhibition, but in this chapter we will focus only on the human telomerase RNA. Here we describe an important genetic method for sensitization of the breast tumor cell line, MCF-7, by using siRNAs that target hTR as a pretreatment to traditional chemotherapeutic treatment, adriamycin and taxol. This approach is especially appealing in that the chemotherapies utilized here are shown to be effective at lower dosages, which will ultimately cause less toxicity to the patient and this sensitization effect within the cells occurs within a much shorter time frame in which there is no lag period.

Using the proposed secondary structure of human telomerase RNA (Chen et al., 2000), we designed 4 target sequences within hTR for RNAi (hTR-T, hTR-1, hTR-2, hTR-3) (Figure 1). All targets are located within the template and pseudoknot domains because these regions have been shown to be vital for telomerase activity due to their interaction with hTERT within the telomerase complex. Sequences were synthesized in the sense and antisense direction individually, approximately 21 nt long, and were then annealed together using a duplex buffer followed by transfection into the MCF-7 breast tumor cells. To assess the consequences of telomerase inhibition after transfection with the siRNAs hTR-T and hTR-1, samples were taken 6, 24, 48 and 72 hours post-transfection for use in a TRAP assay (Figure 2). The only significant reduction detected was with hTR-1 (pseudoknot) at the 72 hour time point. We then tested the ability of the second set of siRNA to inhbit telomerase. The synthetics were transfected into the cells, but in order to optimize knock-down of telomerase, we used three different concentrations for the siRNAs, hTR-2 and hTR-3 (Figure 3), and samples were taken for TRAP 1-3 days post-transfection. We found high levels of telomerase inhibition at all concentrations using the hTR-2 siRNA with the most effective inhibition of telomerase at 90% for 150pmol, which was maintained at each time point. Similarly, with the hTR-3 siRNA, the most efficient concentration of siRNA was 150 pmol with the maximal knockdown at 94%, but at lower concentrations (50 and 100 pmol) inhibition was not as significant or consistent as compared to the hTR-2 siRNA.

Our next goal was to assess if treatment with combinations of siRNAs together produced an even greater inhibition of telomerase than individually. Cells were transfected with the siRNAs (hTR-2 and hTR-3; 150pmol) individually or together, for comparison purposes, into the MCF-7 cells with samples for TRAP analysis and RT-PCR taken 1-3 days post-transfection. We observed high levels of knockdown with the siRNAs in all of the transfected cell lines (Figure 4) and found the use of the hTR-2 siRNA alone works the best in that there is a sustained knock-down of telomerase maxing at 96%. However, hTR-3 produces the greatest knockdown at 98% but this was not consistent for each time point. Both siRNAs together (hTR-2/3) provided successful inhibition at 93%, but this appeared to be a less consistent inhibition than with the other individual siRNAs.

We then measured the levels of hTR using RT-PCR in order to determine if the decreased telomerase activity corresponded with a reduction of RNA levels. As compared to the MCF-7 parental samples, there were lower expression levels of hTR in all three combinations (Figure 5). The cells transfected with the hTR-2 siRNA displayed the least amount of hTR followed by hTR-3 and hTR2/3 respectively, thereby showing a correlation of telomerase inhibition and hTR levels.

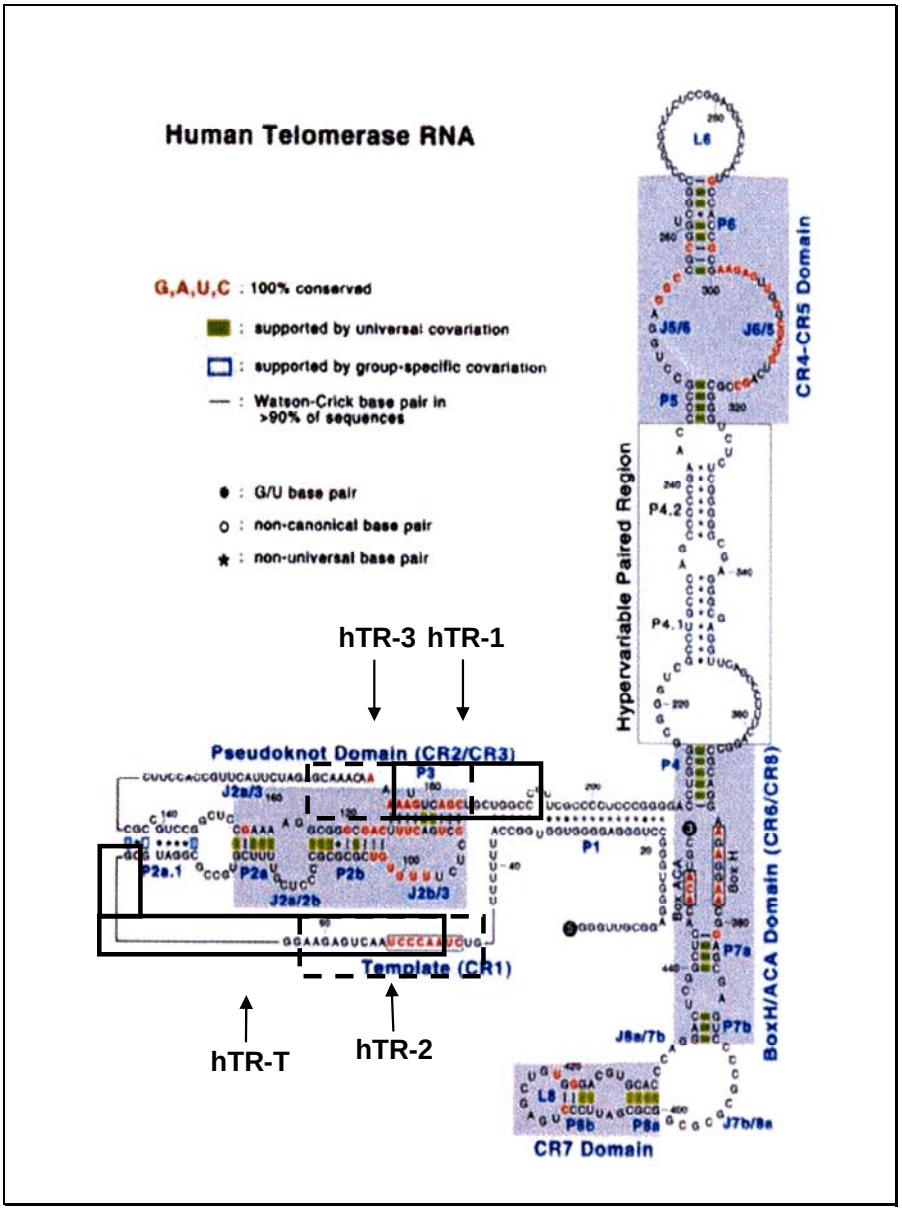

Figure 1: Human Telomerase RNA and siRNA Targets.
Proposed secondary structure of human telomerase RNA (Chen et al., 2000**)** with the five conserved regions enclosed in boxes except for the template region. Also labeled, within the black boxes, are the siRNA target sequences (hTR-T, hTR-1, hTR-2, and hTR-3) with the template and pseudoknot regions respectively.

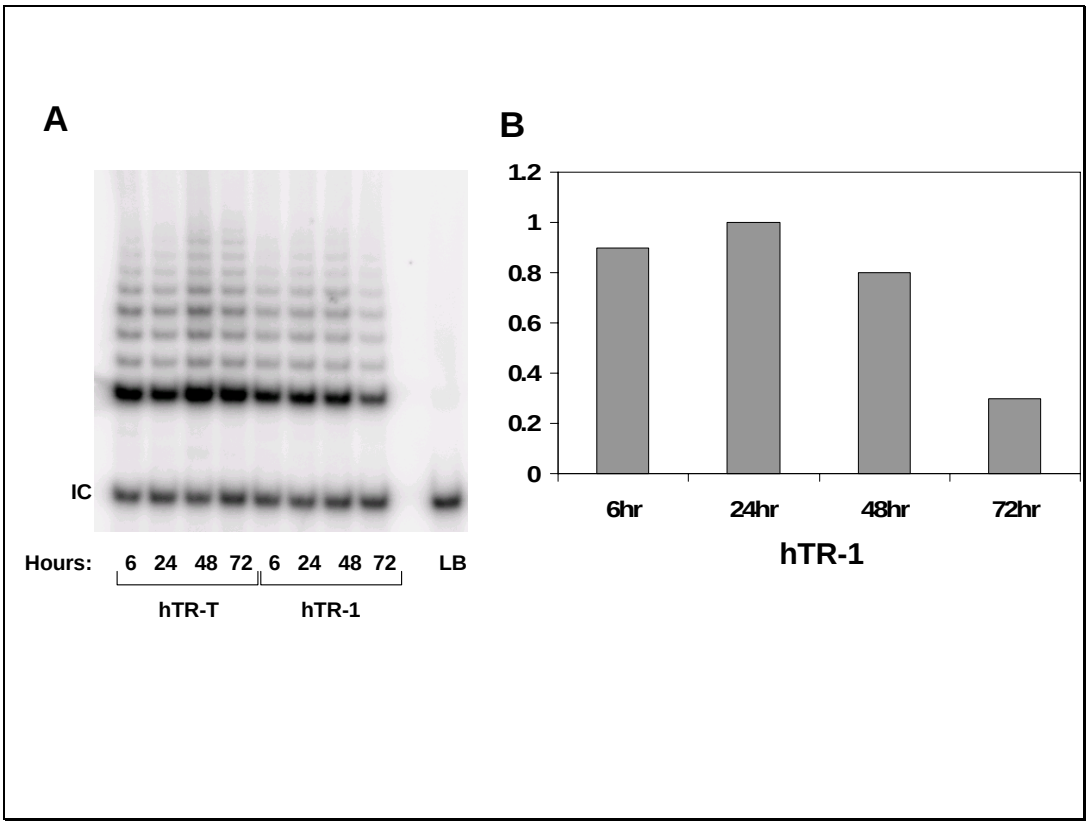

Figure 2: Decline in Telomerase Activity using siRNA Targeted Against hTR. Using the synthetic hTR-T (template) and hTR-1 siRNA (psuedoknot) targeting specific domains of hTR, cells were transfected, harvested and tested for telomerase activity. **A**. Representative telomere repeat amplification protocol (TRAP) showing 250 cell equivalents per sample at various time points is shown. **B.** Quantitation of the relative telomerase activity was accomplished by calculating the ratio of the telomerase ladder to the 36bp internal control (IC), which normalizes sample to sample variation. We observe a reproducible 3 to 4-fold decline in activity after 3 days of treatment.

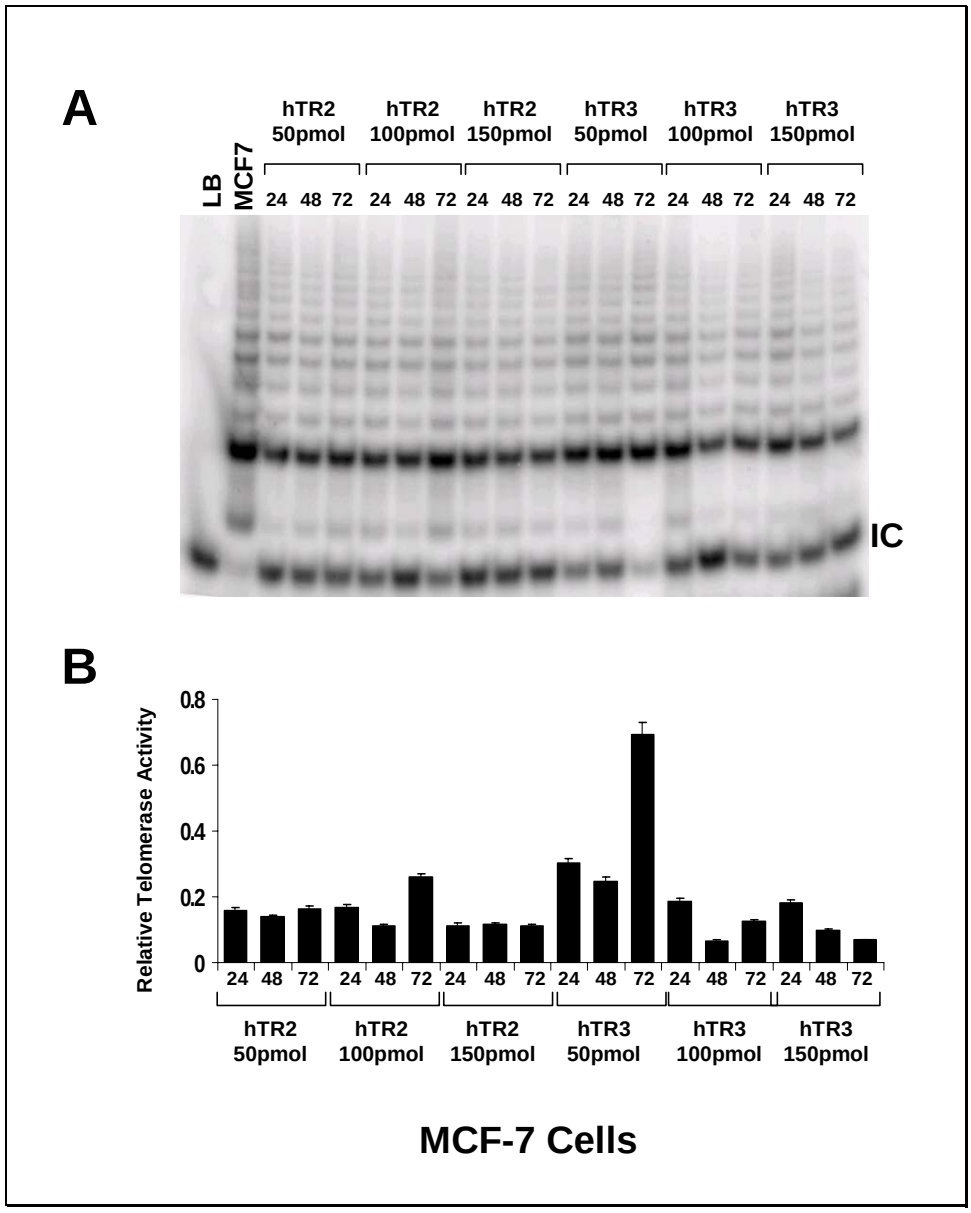

Figure 3: Optimization of Telomerase Inhibition using siRNAs.
A. Representative TRAP assay showing 250 cell equivalents. Synthetic siRNAs, hTR-2 and hTR-3, were transfected into MCF-7 cells at three different concentrations (50, 100, and 150pmol) with samples taken at 24, 48, and 72 hours post-transfection. **B.** Quantitation of the relative telomerase activity was accomplished by calculating the ratio of the telomerase ladder to the 36bp internal control (IC). Each sample was also normalized to the MCF7 cell line and shown as a percent of activity in relation to the MCF-7 cell line. We determined the greatest knockdown of activity occurs at siRNA concentrations of 150 pmol.

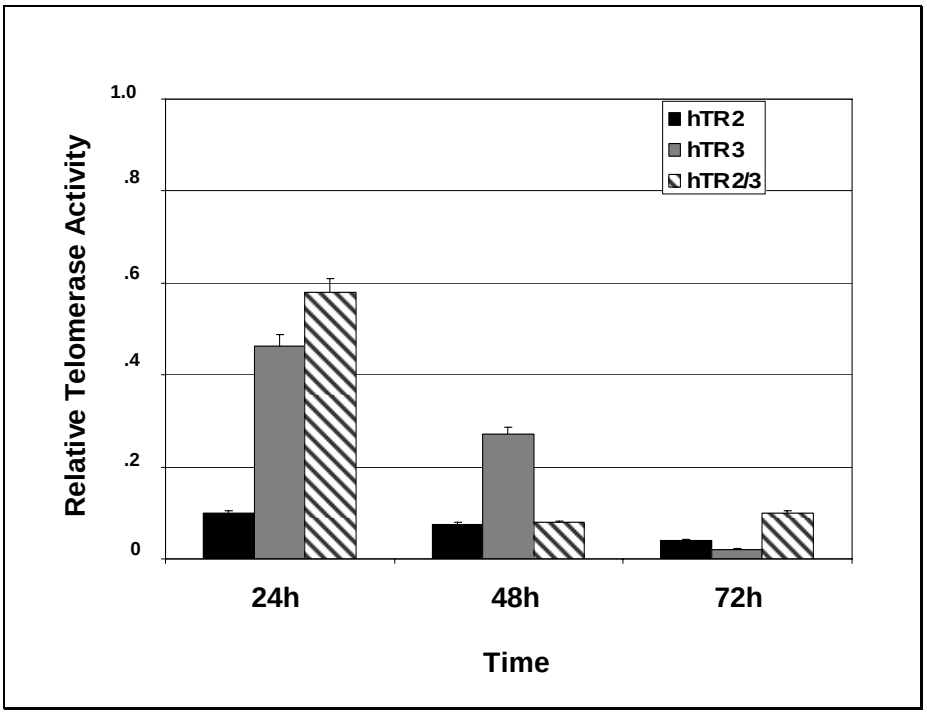

Figure 4. Knockdown of Telomerase Activity using siRNAs Targeting hTR.
Treatment of MCF-7 cells with siRNAs hTR-2 and hTR-3 singularly and in combination at 150pmol concentration with samples taken at 24, 48, and 72 hours post-transfection. Quantitation of the relative telomerase activity was accomplished as stated in Figure11. We determined the greatest and most consistent telomerase inhibition occurs with the hTR2 siRNA.

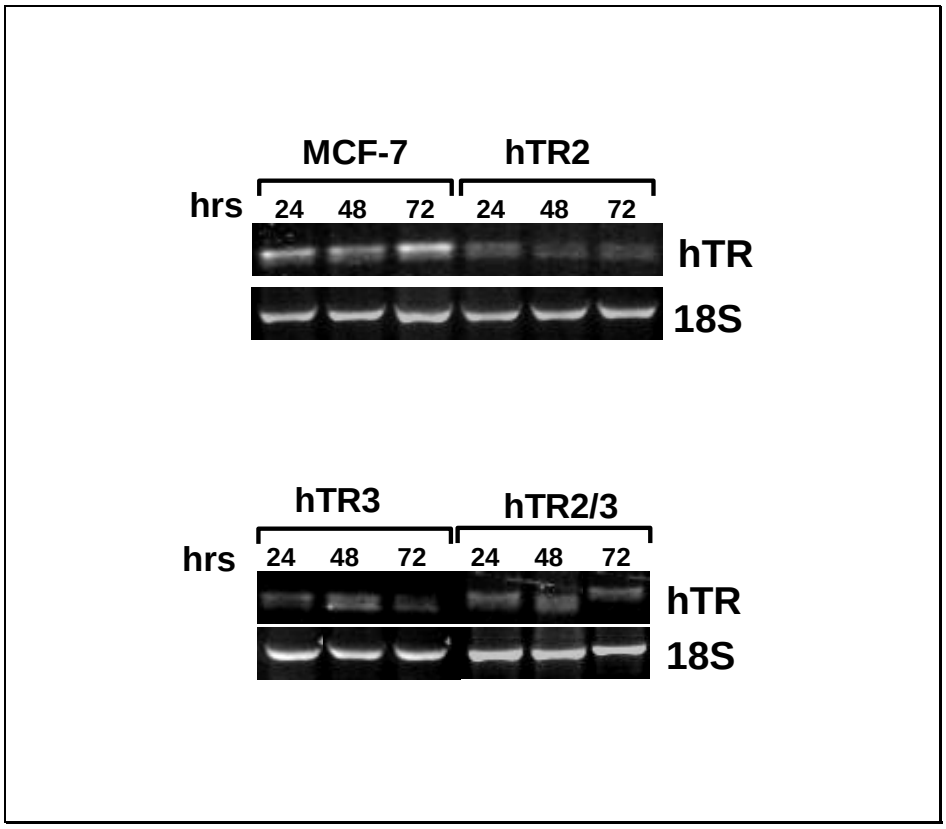

Figure 5. Decreased hTR Expression after Transfection with siRNAs. Treatment of MCF-7 cells with siRNAs hTR-2 and hTR-3, singularly and in combination at 150pmol concentration. RT-PCR was conducted to determine the expression levels of hTR 24, 48, and 72 hours post-transfection. We showed the most stable knock-down of hTR occurs with the hTR2 siRNA.

***Objective #2*: Determine Sensitization of Breast Tumor Cells using siRNA targeting hTR as a Pretreatment to standard chemotherapies.**

Now that significant telomerase inhibition has been established using RNAi we determined sensitization of these cells using chemotherapy. One of the older chemotherapeutic drugs that have been in use for decades for the treatment of a variety of cancers, including breast cancer, is Adriamycin (AdR) or Doxorubicin, an anthracycline antibiotic and topoisomerase II inhibitor. Adriamycin functions by blocking replication in that it stabilizes the topoisomerase II, which unwinds the DNA by breaking one strand, and DNA complex thereby preventing the DNA double helix from being resealed or ligated. Treatment with AdR has been shown to cause apoptotic death in a variety of tumor cell lines other than breast, such as human and murine leukemia cell lines (Ling et al. 1993; Zaleskis et al. 1994; Jaffrezou et al. 1996).

The siRNA hTR-2 produced the most effective inhibition of telomerase; therefore, this was the only means of a pre-treatment within the MCF-7 breast cancer cell line utilized for these experiments. The cells were transfected with hTR-2 siRNA (150pmol) and allowed to grow for three days in order for sufficient and sustained knock-down of telomerase to occur. An acute AdR treatment at various concentrations for 2 hours was given to the cells with samples taken 2 and 4 days post-AdR. Growth of the cell lines was calculated to ascertain if pre-treatment with the siRNA followed by AdR produced a growth effect (Figure 6). No difference as compared to the control MCF-7 cells was observed at day 2, but by day 4 cells had significantly slowed growth in the cells treated with the siRNA hTR-2 at all concentrations except for 0.75μM.

In order to determine if senescence could be induced regardless of telomerase activity or telomere length, we quantitated cellular senescence in the cells post-treatment with AdR. The senescent phenotype is illustrated by a viable cell that is incapable of responding to proliferation or apoptotic signals. Senescence is further characterized by flattened and enlarged cell morphology as well as by increased levels of the biomarker, senescent-associated beta galactosidase (SA-β-gal) (Lee et al. 2006). We detected levels of SA-β-gal in cells using the chromogenic substrate X-gal, which stains the cells blue when activated at pH 6. Significantly elevated levels of senescence were found by day 2 in all cell lines as compared to the MCF-7 control cells regardless of the concentration of AdR administered (Figure 6). Other studies have shown induction of senescence in MCF-7 cells 3-4 days after exposure to the clinically relevant dosage of 1μM AdR (Elmore et al. 2002). Therefore, generation of such high levels of senescence 2 days post-treatment and at concentrations of AdR as low as 0.02μM is extremely significant, revealing definite sensitization of those cells to AdR and providing further support that the senescence response is telomere length independent.

Lastly, we examined the percent apoptotic cells after treatment with AdR using the TUNEL assay, in order to determine how apoptosis was affected by pre-treatment with hTR-2 siRNAs and subsequently, AdR (Figure 7). Apoptosis, or programmed cell death, is characterized within the cell by several phenotypes, some of which include cell shrinkage, nuclear fragmentation, condensation of the nuclei and cytoplasm, as well as loss of cell surface structures. In order for this process to occur, several signaling pathways must be activated, which in turn specifically activate a group of proteases or

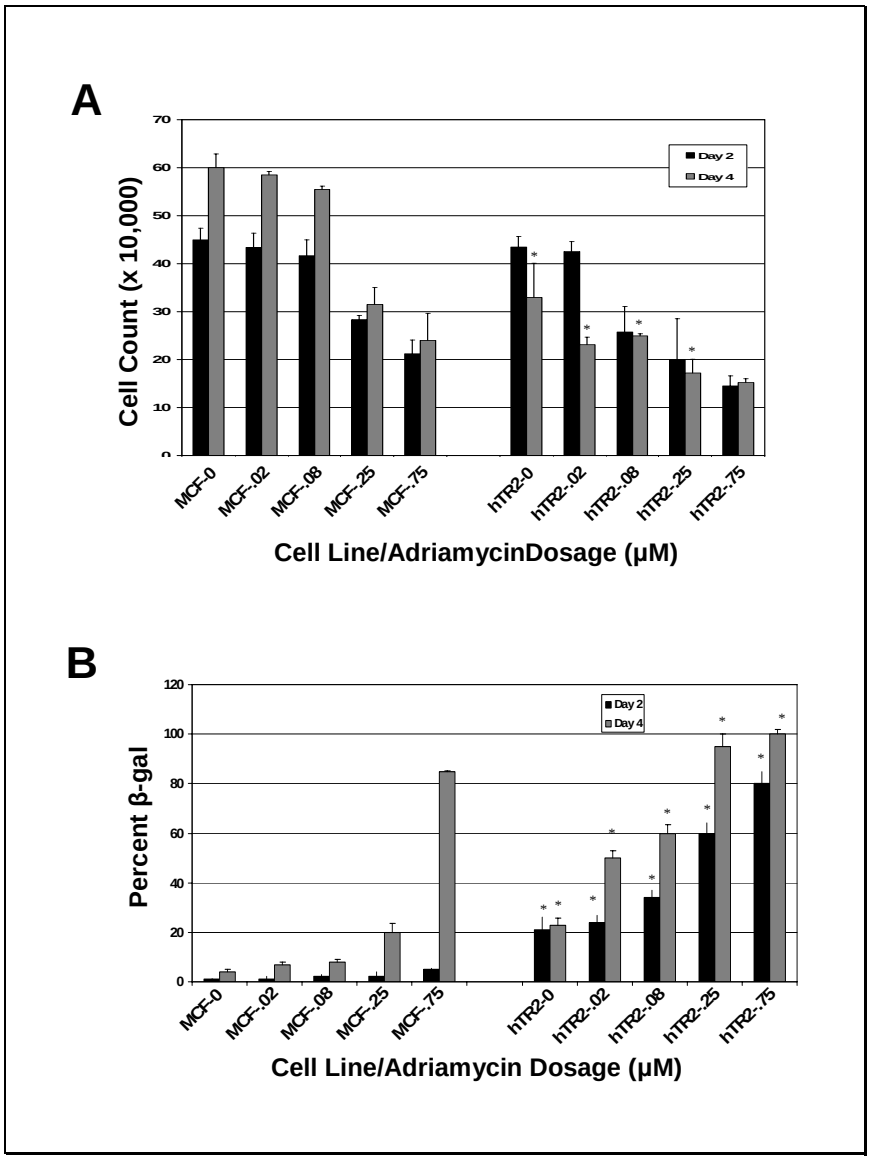

Figure 6. Decreased Growth and Increased Senescence in MCF-7 Cells Post-Treatment with Synthetic siRNAs and Adriamycin (AdR). Cells were transfected with the synthetic hTR2 siRNA (150pmol). Three days later, cells were treated with various concentrations of AdR, and samples were then taken 2 and 4 days post-treatment with AdR. **A.** Cell numbers were counted using a hemocytometer and samples were done in duplicate. By day 4 cell growth had slowed significantly, as compared with controls, at dosages lower than clinically relevant. **B.** Cells were fixed and incubated overnight with a β-gal staining solution. At day 2, cells transfected with hTR2 siRNA had significantly higher levels of senescence at all AdR concentrations. Columns represent the calculated mean from three representative fields of 100 cells; bars, SD. Significance for both graphs was determined by a two-tailed t-test and displayed on the graphs. *, $P < 0.05$.

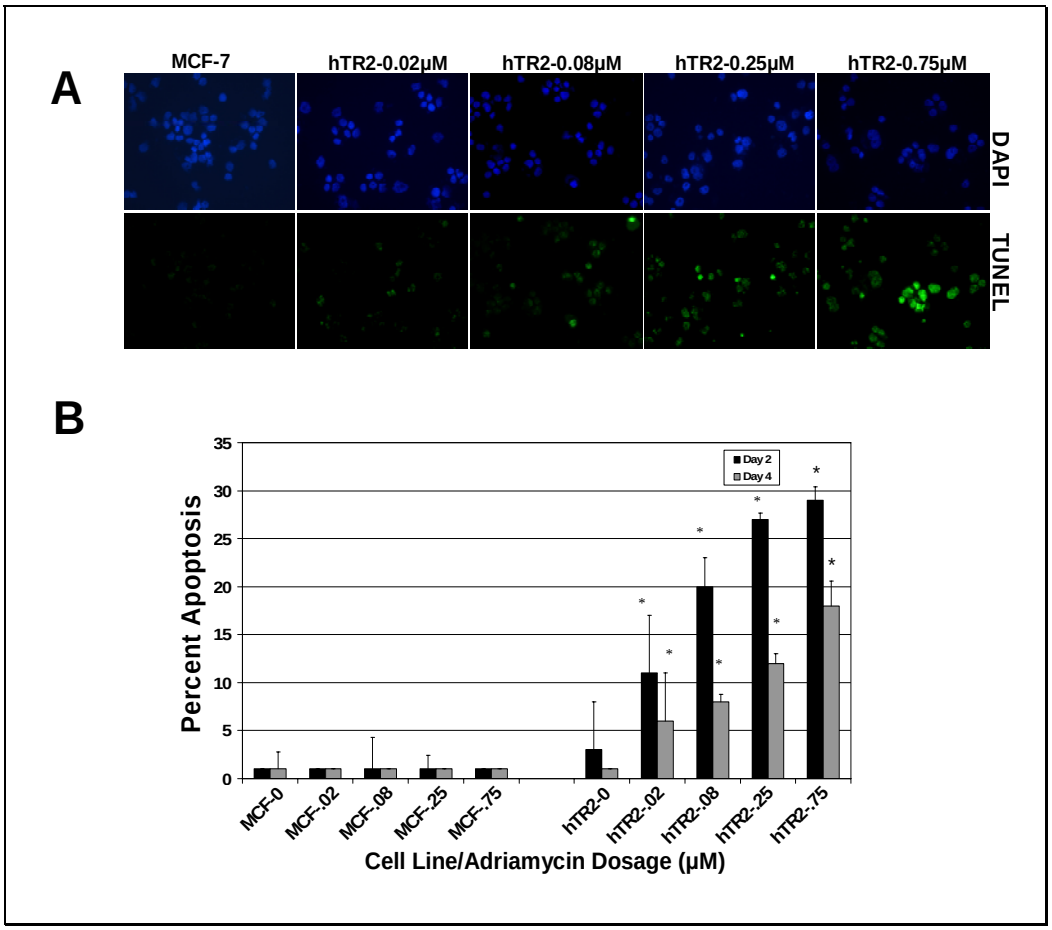

Figure 7. TUNEL Staining for Apoptosis in MCF-7 Cells Post-Treatment with Synthetic siRNAs and Adriamycin (AdR). Cells were transfected with the hTR-2 siRNA construct. Three days later, cells were treated with various concentrations of AdR. Samples were then taken 2 and 4 days post-treatment. **A.** At day 4, increased apoptosis was evident. The DAPI (blue) stains the nucleus and the Fluoroscein 12-dUTP stains the cells positive for apoptosis (green). **B.** Quantitation of TUNEL assay revealing, at the concentrations of 0.02-0.75 μM AdR were the levels of cell death appreciably higher at days 2 and 4 than the controls. Columns represent the calculated mean from 3 representative fields of 100 cells; bars, SD; *, $P < 0.05$.

enzymes known as the caspases. Ultimately, caspase activation leads to the cleavage of DNA into <200bp fragments, which can then be detected using the TUNEL assay. Specifically, cells are fixed and incubated with fluoroscein 12-dUTP, which is transferred onto the ends of the fragmented DNA by a terminal deoxynucleotidyl transferase. Positive cells are stained intensely green, which is judged against the DAPI staining of the same set of cells in order to compare the nuclear staining and the TUNEL positive cells, as well as confirm that a positive TUNEL signal is in fact a cell and not debris. As compared to the control MCF-7 cell line, we found increased incidences of apoptosis in all samples except the untreated cell lines containing only hTR-2 siRNA. So from exposure to AdR at dosages as low as 0.02μM, a 9-fold difference to the clinically relevant dosage of 0.75μM, the hTR-2/MCF-7 cells underwent cell death at significantly higher quantities than the control cell lines. Other studies have shown that MCF-7 cells will only undergo senescence after treatment with AdR because these cells are p53 positive, but we provide evidence that apoptosis does occur at clinically relevant dosages of AdR (Elmore et al. 2002).

As a result, we wanted to assess levels of p53 in the cells to determine how the cells responded to the chemotherapeutic treatments. We transfected cells with either hTR-2 or a scrambled siRNA shown to have no effect within the cell, and 3 days later the cells were treated with various concentrations of AdR for two hours (Figure 8). Samples for western blots were taken 6 and 24 hours post-treatment to assess protein levels. Constituitively, p53 levels were slightly higher in the hTR-2 siRNA cell lines than those found in the scrambled siRNA control cell lines, suggesting a possible increase in DNA damage after telomerase inhibition. As expected, there was an up-regulation of p53 and therefore, p21 activated by p53, after treatment with AdR at all concentrations at the 6 hour time point. However, by 24 hours post-treatment, the elevated levels of p53 had decreased back to normal. In both cell lines, there were no real differences in regulation of p53 during this comparison.

Another mainstay in the treatment of breast cancer, early stage and metastatic, is the chemotherapeutic drug known as Taxol or paclitaxel, which is a member of the group of taxanes (Jemal et al. 2005). Taxol binds to and promotes the formation of mitotic splindle microtubules and then stabilizes them, thereby preventing depolymerization during cellular division (Horwitz et al. 1993; Rao et al. 1995). As a result, the segregation of the sister chromatids is also prevented and the cells are blocked at G2/M phase of the cell cycle. The destruction of the normal dynamic reorganization of the microtubule network, as well as the block in the cell cycle, ultimately leads to cell death (Milross et al. 1996; Yvon et al. 1999).

In order to compare sensitization of MCF-7 breast tumor cells to different types of chemotherapeutic drugs, we again utilized the synthetic siRNA hTR-2 as a pretreatment as it produced the most effective inhibition of telomerase and the same experimental method. Briefly, cells were transfected with hTR-2 siRNA (150pmol) and allowed to grow for 3 days. An acute taxol treatment at various concentrations for 2 hours was administered to the cells, followed by samples harvested at 2 and 4 days post-treatment. The first time the experiment was conducted the range of taxol (0.0-1.5μM/L) was too high in that the toxicity of the drug caused no significant differences between the hTR-2 and MCF-7 cell lines because neither cell line grew as shown in the day 2 samples and even less growth was seen by day 4 (Figure 9). Therefore, the experiment was repeated.

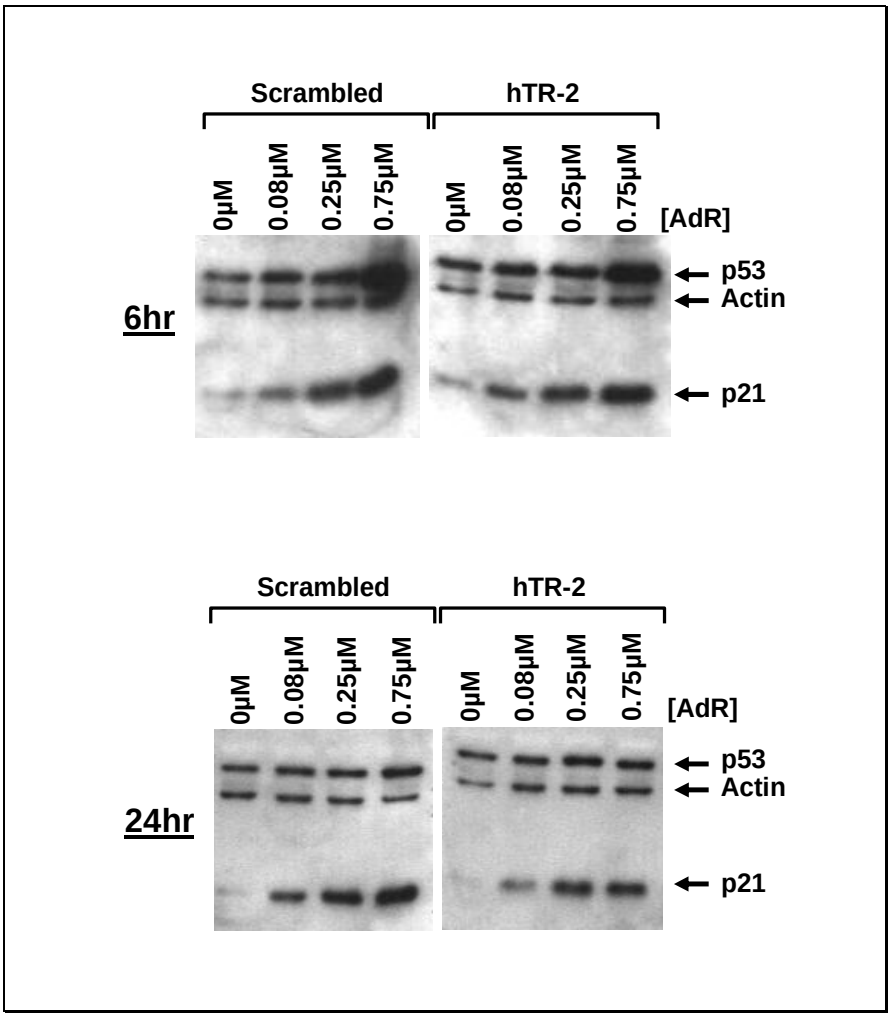

Figure 8. Induction of DNA Damaging Proteins after Treatment with Adriamycin. After transfection with the hTR-2 siRNAs and scrambled siRNAs, the MCF-7 cells were treated with a 2 hour acute dose with various concentrations of AdR. Samples were taken 6 and 24 hours post-treatment, cell pellets were collected, and 15μg of total protein lysate was subject to Western analysis. Immunoblots were probed with anti-p53, anti-p21, and anti-actin as a loading control.

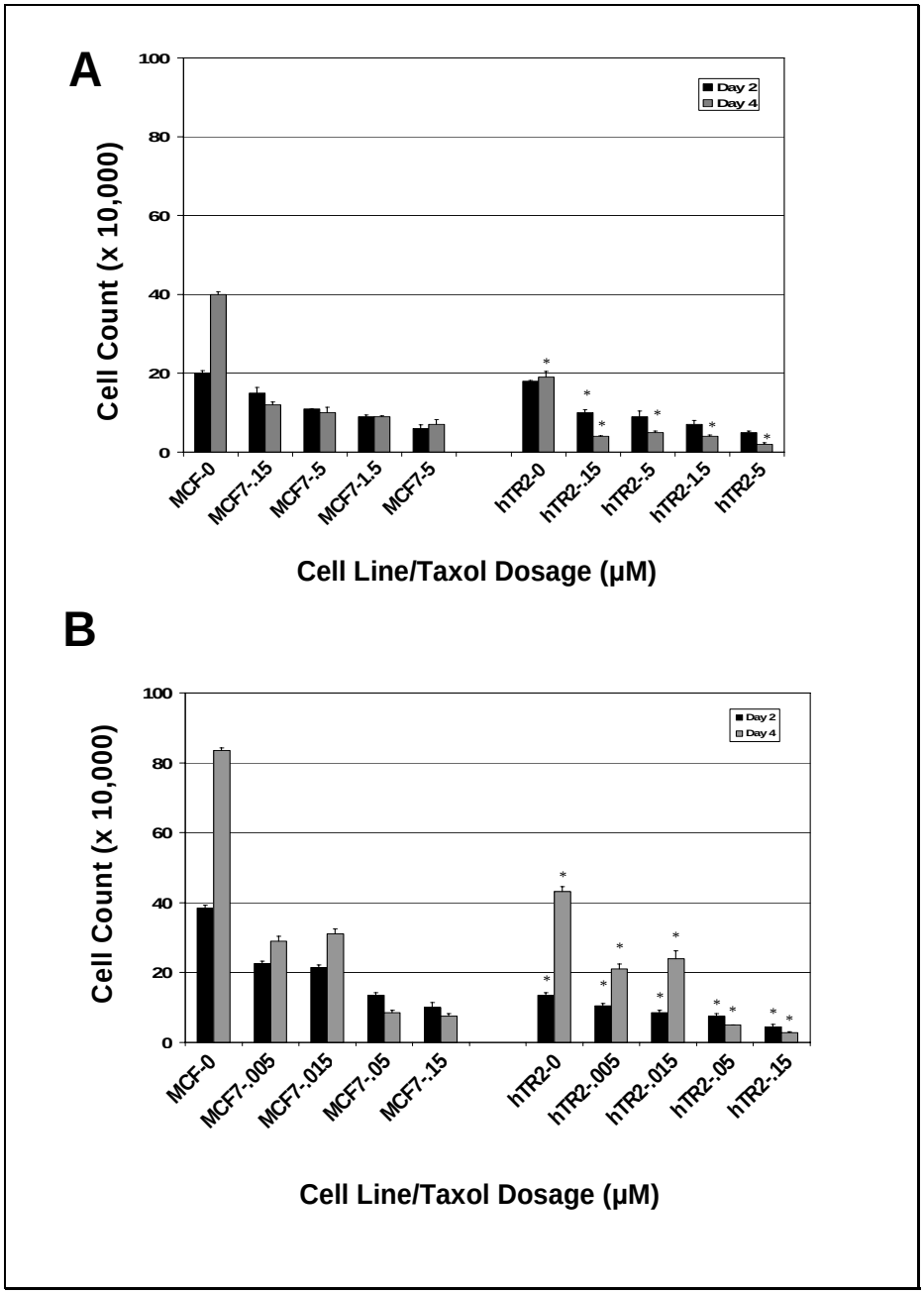

Figure 9. Growth of MCF-7 Cells Post-Treatment with Synthetic siRNAs and Taxol. Cells were transfected with the hTR2 siRNA construct. Three days later, cells were treated with various concentrations of taxol. Samples were then taken 2 and 4 days post-treatment. Cell numbers were in duplicate. The higher taxol **(A)** concentrations were too toxic for both the control and siRNA cell lines, compared to lower (B) range of concentrations. Columns represent the calculated mean from three representative fields of 100 cells; bars, SD; *, $P < 0.05$.
with a lower range of taxol dosages (0.0-0.15μM). The control cell lines grew at a steady rate, and the MCF-7/hTR-2 cells lines had significantly slower growth at every taxol concentration compared to controls.

We then evaluated the levels of senescence to assess how taxol affects senescence after telomerase is inhibited. We found at day 2 post-treatment, all cell lines revealed significantly high levels of senescence regardless of the concentration of taxol administered (Figure 10). However, by day 4 only taxol concentrations from 0.0-0.015 μM displayed considerably elevated levels of senescence, implying the higher drug concentrations of 0.05 and 0.15 μM produced similar results as the MCF-7 control cell lines and sensitization only occurred at the lower two dosages.

Finally, we assessed levels of cell death to determine the effects of taxol post-treatment with the siRNA hTR-2. Apoptosis increased significantly for samples taken on both days, especially on day 4 when the percent cell death ranged from 35-50%, but only for the drug concentrations of 0.015-0.15 μM taxol (Figure 10). Other researchers have shown apoptosis induction by taxol treatment in MCF-7 cells by 2 days at dosages of .010 μM, but in that study, taxol treatment was chronic (for the entire 48 hours), whereas our treatment was acute (for only two hours) (Wu et al. 2006).

Genetic Inhibition of hTERT and Sensitization of MCF-7 Cells

***Objective #3*: Maintain stable suppression of hTERT using RNA interference with high levels of telomerase inhibition.**

Together with the hTR templating RNA, the other key component of telomerase is hTERT, which must be activated in order for telomerase activity to be restored in human malignancies, making this protein a promising target for cancer therapy. Other studies have attempted to target hTERT in a chronic fashion and inhibit telomerase using a variety of strategies including ribozymes, peptide nucleic acids, and antisense oligonucleotides, as well as combining these strategies with chemotherapy (Hao et al. 2005; Kraemer et al. 2004). However, here we show that telomerase can be inhibited in breast tumor cells, transiently using RNAi and then sensitization of these cells occurs rapidly post-treatment with chemotherapeutic drugs.

As seen in Figure 11, hTERT is composed of two very important features, namely, the telomerase specific motif T specific for telomerases, as well as the seven reverse transcriptase (RT) motifs (1, 2, A, B', C, D, and E) that are conserved among the family of reverse transcriptases (Nakamura et al. 1997). RNAi target sequences for hTERT were acquired (Nakamura et al. 2005; Masutomi et al. 2003), followed by synthesis in the sense and antisense direction individually, approximately 21 nt long. Using duplex buffer, the oligonucleotides were then annealed followed by transfection into the MCF-7 breast tumor cells. One of the hTERT siRNA targets is located within the RT motif 2 or exon 5, and the other sequence lies outside of the conserved RT region in exon 14. To assess the expression levels of hTERT after inhibition with the siRNAs (hTERT-1, hTERT-2, hTR-2, and hTR-3), either singularly or in various combinations, samples were taken 24, 48 and 72 hours post-transfection for use in RT-PCR (Figure 11). As compared to the MCF-7 control cell line, significant decreases in hTERT RNA were only observed post-transfection with the siRNAs hTERT-1, hTERT-1/2 and Pooled, which is all four siRNAs together. Variable knock-down of hTERT RNA was shown when hTERT-1 was utilized in combination with hTR-2 or hTR-3. The siRNA combinations that did not produce consistent decreased hTERT RNA levels were hTERT-2, as well as hTERT-2 with either hTR-2 or hTR-3 indicating that hTERT-2 is not the most efficient siRNA for use in telomerase inhibition. Interestingly, it seems that the combination of hTERT-1 and -2 caused a significant and immediate decrease in hTERT, which is consistent with the hTERT-2 having an immediate effect and hTERT-1 having a more gradual but pronounced effect (Figure 11). However, the primer set utilized for detecting hTERT amplifies a region located upstream of both hTERT target sequences, which might have caused the variability seen in the hTERT RNA levels.

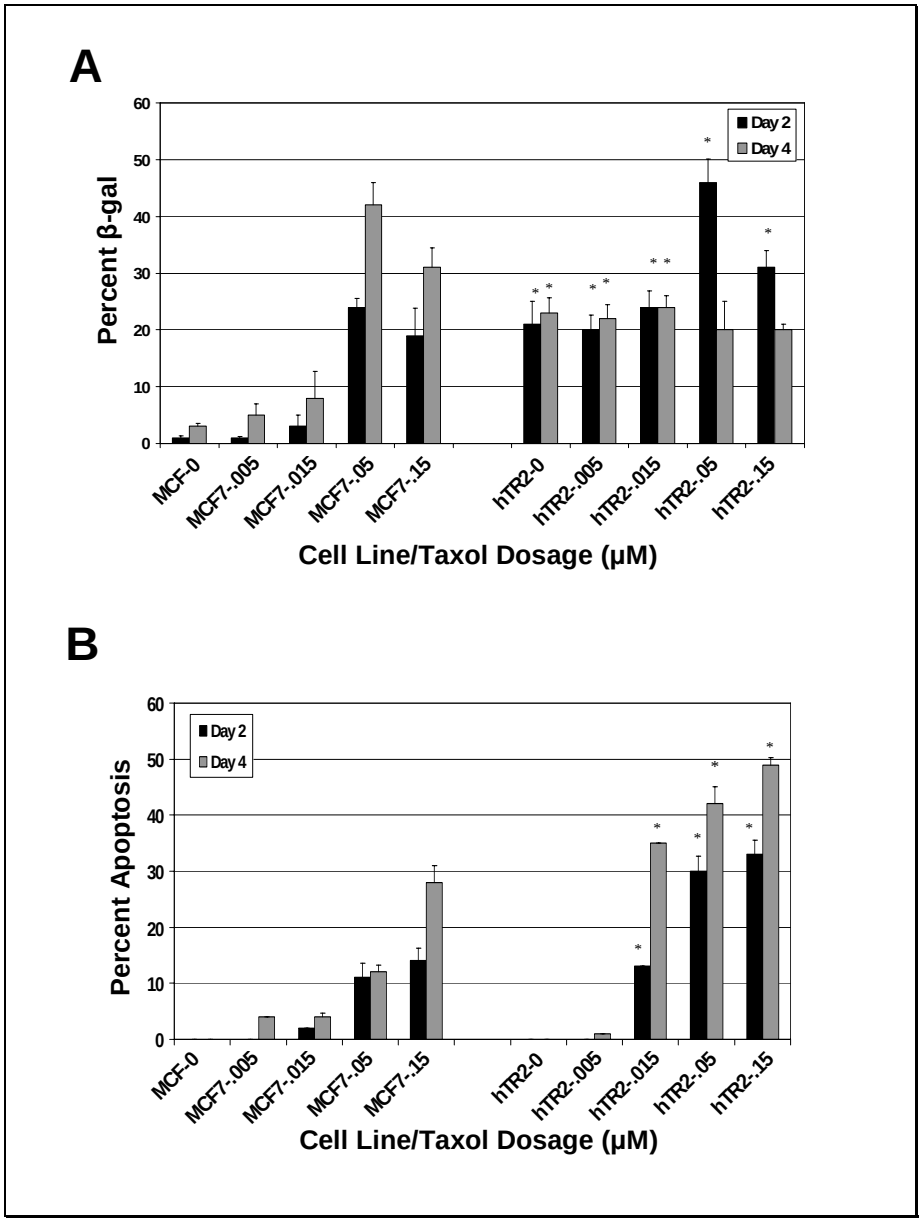

Figure 10. Cells Treated with Synthetic siRNAs and Taxol Showed Increased Sensitivity to Apoptosis. Experimental method is the same as in Figure 17. **A.** Cells were in fixed and incubated overnight with a β-gal staining solution. Differences were observed in senescence were seen in day 2 but only at taxol concentrations from 0.0-0.015 μM on day 4. B. TUNEL assay showing percent apoptosis and levels were considerably higher in the cells treated with 0.015-0.15 μM Taxol on both days as compared to MCF-7 cells. Columns mean from three representative fields of 100 cells; bars, SD; *, $P < 0.05$.

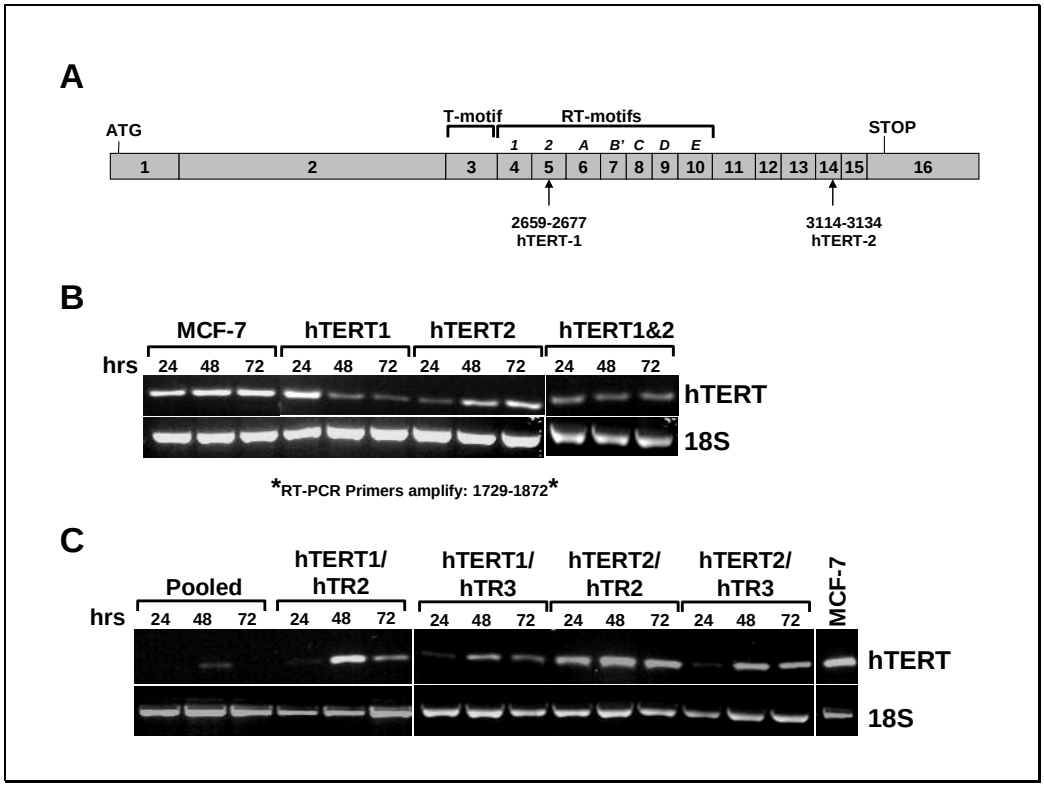

Figure 11. Schematic of the hTERT Gene and Knockdown of hTERT RNA Levels using Synthetic siRNAs. A. Linear schematic of the hTERT gene with the T-motif as well as the seven conserved RT-motifs labeled above the gene. Also labeled are the siRNA target sequences. **B.** Treatment of MCF-7 cells with siRNAs targeting hTERT only (150pmol). RT-PCR was conducted to determine the expression levels of hTERT 24, 48, and 72 hours post-transfection. We showed the hTERT1 siRNA caused the most stable knockdown. **C.** RT-PCR of MCF-7 cells after treatment of different combinations of siRNAs targeting hTERT and hTR, which revealed the Pooled siRNAs (hTR2, hTR3, hTERT1, and hTERT2) to produce the most efficient inhibition of hTERT expression. The location of the sequence amplified by the primers in the RT-PCR experiments is located below the gel in part **B.**

Because reduction of hTERT mRNA was confirmed within the breast tumor cells, TRAP samples were collected 24, 48 and 72 hours post-transfection, to assess telomerase activity after inhibition with the siRNAs. In the first analysis, cell lines containing the siRNAs targeting hTERT plus the Pooled siRNAs were tested for telomerase (Figure 12). Telomerase activity was highly decreased in all of the cell lines with the hTERT-1 siRNA, which produced the most effective levels of inhibition at 98% by day 3. On the other hand, hTERT-2 by day 1 showed 93% inhibition but this telomerase activity increased with each sample and inhibition dropped to 80% by day 3. Taken together, these results are consistent with the RNA knockdown shown in Figure 25. The Pooled siRNA and hTERT-1 and -2 both displayed consistent amounts of knockdown among the samples at 90% and 81%, respectively.

Our second study analyzed telomerase inhibition after transfection with the different combinations of the hTERT and hTR targeting siRNAs together (Figure 13). The percent inhibition of telomerase that most corresponded with the previous study was found after transfection with hTERT-1/hTR-2 together and maximal levels of knockdown of telomerase activity was 98%. However, when hTERT-1 and hTR-3 were transfected together, the levels of telomerase activity progressively decreased with each sample taken ranging from 65-91%. Similarly, with hTERT-2/hTR-3 the levels of telomerase activity gradually decreased but the scope of inhibition was 0-66%. With the last combination of siRNAs, hTERT-2/hTR-2, the telomerase activity levels as seen on day 1 were the lowest and then grew larger with every sample so the percent inhibition from 24 to 72 hours went from 82% to 62%. It is important to note here that although the telomerase ladder is visible in each lane (even with a 98% reduction in quantifiable activity levels); the quantification is done by taking a ratio of the telomerase ladder to the internal control, which is amplified in a semi-conservative manner. Thus, even though activity is detected, it is substantially reduced after siRNA knockdown of telomerase components.

Significant decreases in telomerase activity were found even in those samples where hTERT RNA levels were not diminished, which could be partially due to the primer set used for the RT-PCR. These primers were the original set used by Feng et al. (1995), which are not near the site of siRNA complementation. As such, a more appropriate primer set was designed to encompass the region(s) of siRNA knockdown. The primers amplified the region that technically would be cut by the hTERT-1 siRNA (Figure 14). Therefore, RT-PCR was repeated on the same cells containing siRNA targeting hTERT. As compared to the MCF-7 control, knockdown of hTERT RNA was observed in all samples, which confirmed the capability of the hTERT-1 siRNA to cause degradation of hTERT mRNA. Interestingly, we also saw a slight decrease in the levels of hTERT post-treatment with the hTERT-2 siRNA.

We also wanted to investigate the effects of RNAi using different combinations of hTERT siRNAs on the levels of hTR. The RNA levels within the cells transfected with siRNA targeting both hTERT and hTR is shown in Figure 15. We found high levels of hTR knockdown in the cell lines with the hTR-2 siRNA but not with hTR-3 regardless of the hTERT siRNA also used, indicating the template region of hTR is a more efficient target of hTR than the pseudoknot region. Another study has shown that the hTERT protein functions to stabilize hTR in that the steady-state hTR levels increase with increased expression of endogenous hTERT (Yi et al. 1999). Therefore, we wanted to determine if the reverse was true, that a decline in hTERT would cause a decline in hTR levels. Using RT-PCR, we found a slight decrease in hTR levels after day 1 that was maintained for the 2 and 3 day samples (Figure 15), suggesting that hTERT is capable of stabilizing hTR as shown previously (Yi et al., 1999).

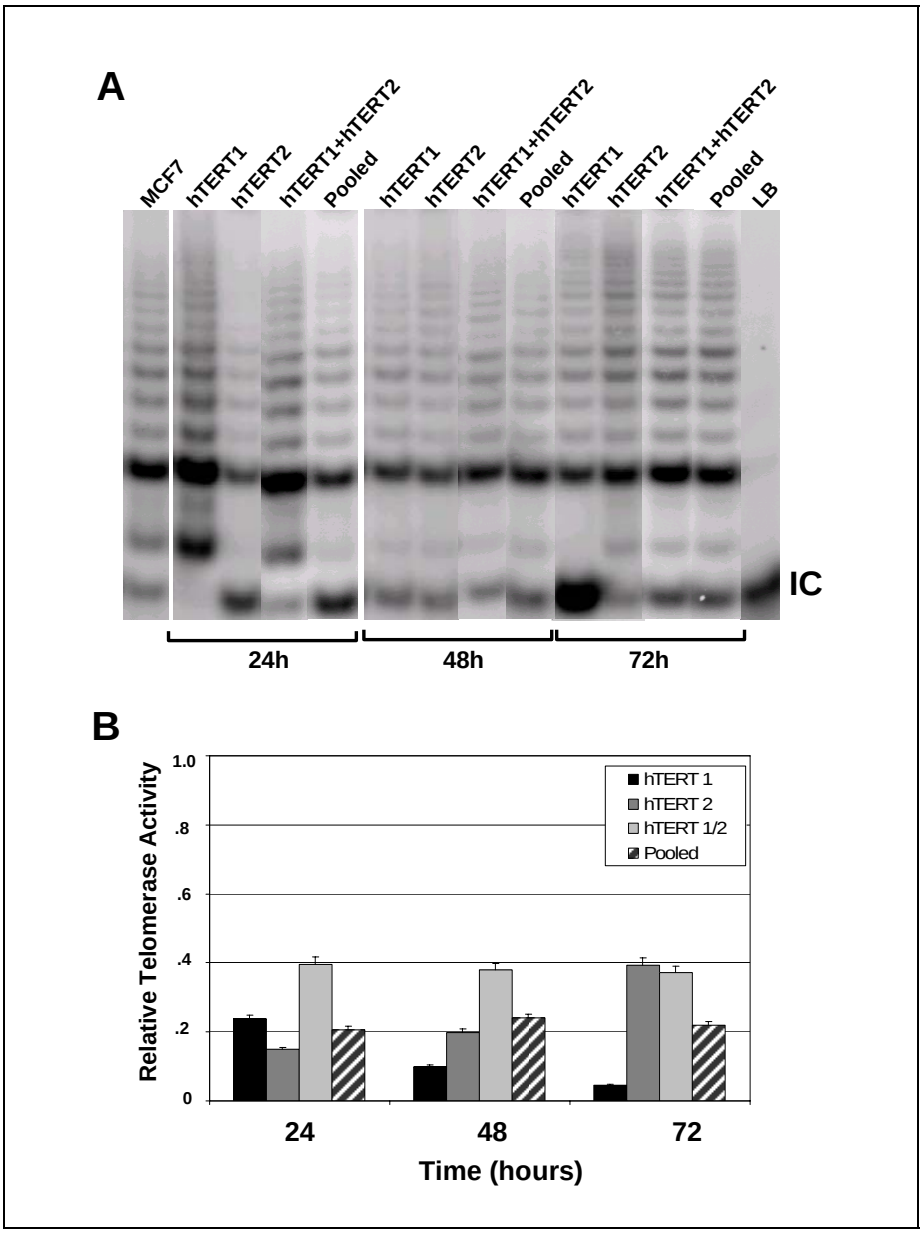

Figure 12. Knockdown of Telomerase Activity using siRNAs Targeting hTERT.
Treatment of MCF-7 cells with siRNAs singularly and in combination at 150pmol concentration with samples taken at 24, 48, and 72 hours post-transfection. **A.** Representative TRAP assay using 250 cell equivalents. **B.** Quantitation of the relative telomerase activity was accomplished as stated in Figure 11. The greatest knockdown of activity was determined to occur with hTERT-1 while the most consistent telomerase inhibition occurs with the Pooled siRNAs (hTERT-1, hTERT-2, hTR-2, and hTR-3).

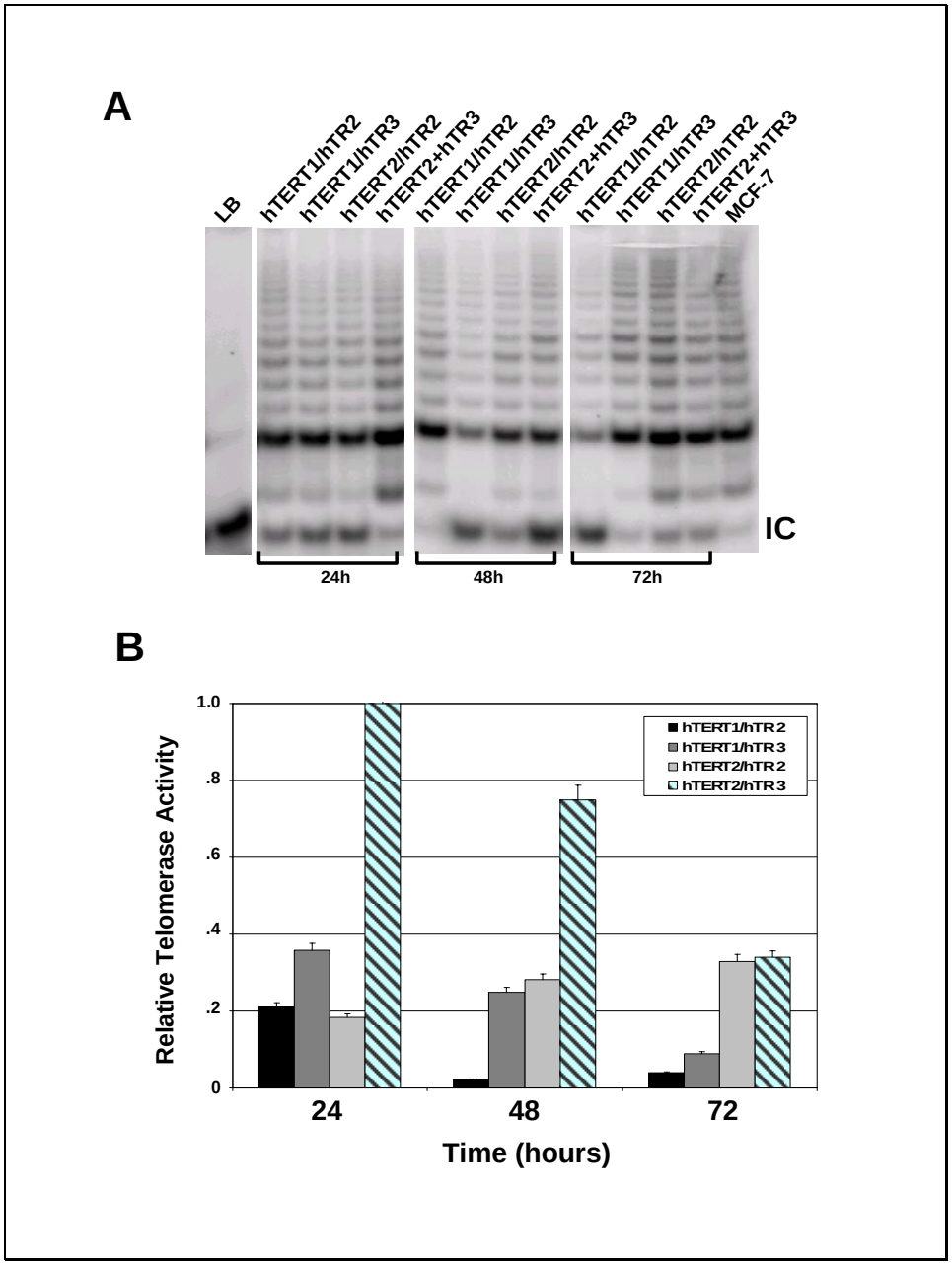

Figure 13. Telomerase Inhbition using siRNAs Targeting hTERT and hTR Simultaneously, as outlined in Figure 26. **A.** Representative TRAP assay showing 250 cell equivalents. **B.** Quantitation of relative telomerase activity was accomplished as stated in Figure 11. When knocking down both hTERT and hTR, the largest inhibition was observed with the combination of hTERT-1 and hTR-2 siRNAs. The other synthetic siRNA combinations also reduced telomerase activity to varying degrees.

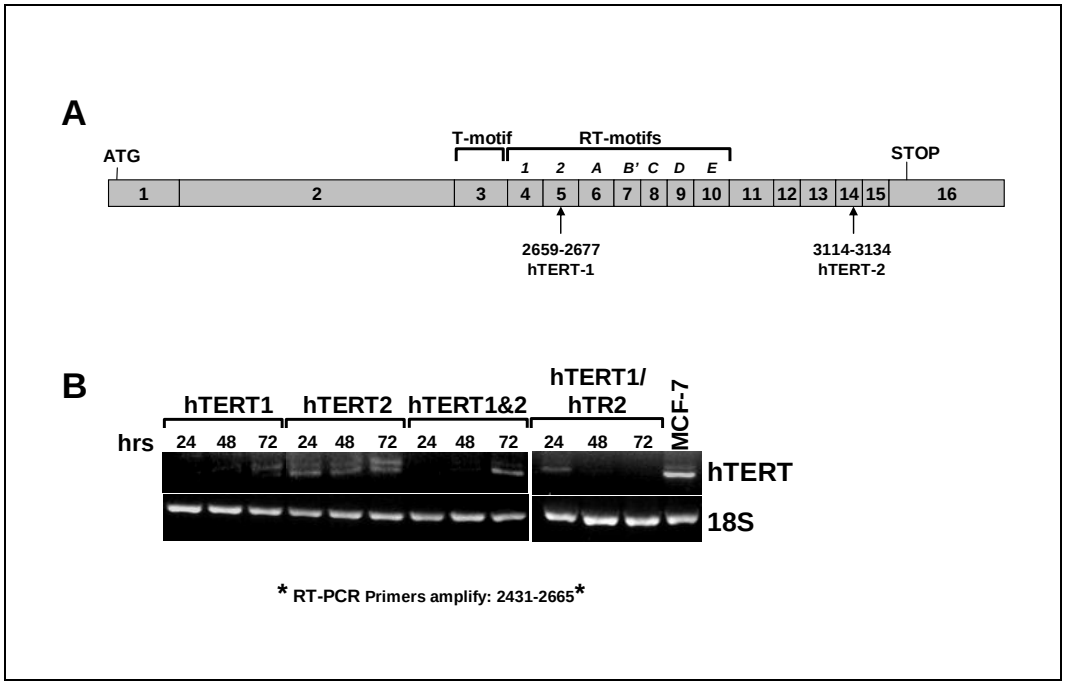

Figure 14. Schematic of the hTERT Gene and Knockdown of Telomerase RNA Levels using Synthetic siRNAs. A. Linear schematic of the hTERT gene with the T-motif as well as the seven conserved RT-motifs labeled above the gene. **B.** Transfection of MCF-7 cells with siRNAs targeting hTERT and hTR (150pmol). RT-PCR was conducted to determine the expression levels of hTERT 24, 48, and 72 hours post-transfection with primers that enclose the region targeted by the hTERT1 siRNA. We showed a decrease in the expression of hTERT with all of the siRNAs, singularly or in combination, except hTERT2 siRNA. The location of the sequence amplified by the primers in the RT-PCR experiments is located below the gel.

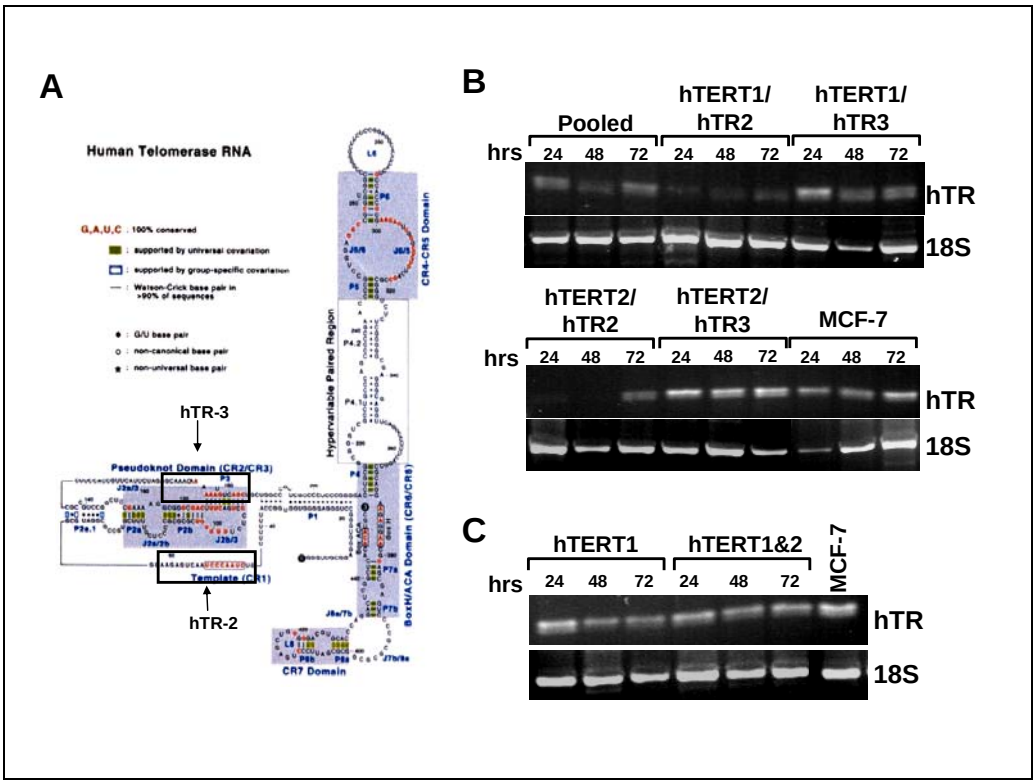

Figure 15. Decreases in hTR levels after Treatment with Combinations of synthetic siRNAs Targeting Telomerase. A. Schematic of the proposed secondary structure of hTR (Chen et al., 2000**)** and the siRNA target sequences, hTR-2 and hTR-3, within the template and pseudoknot regions, are identified within the black boxes**. B.** RT-PCR of MCF-7 cells after treatment of the assorted combinations of siRNAs targeting hTERT and hTR, which indicated the hTERT-1/hTR-2 and hTERT-2/hTR-2 to generate the greatest inhibition of hTR RNA levels. **C.** RT-PCR of MCF-7 cells after treatment with hTERT-1 and hTERT-1 and -2 in order to determine hTR levels were affected by knocking down hTERT levels.

***Objective #4*: Chemosensitization of Breast Tumor Cells After siRNA Silencing of hTERT**

In order to provide the most comprehensive coverage of sensitization using knockdown of hTERT, we utilized three sets of siRNAs (hTERT-1, hTERT-2, and Pooled) as a pre-treatment to exposure to Adriamycin (AdR). The cells were transfected with the siRNAs (150pmol each) and allowed to grow for 3 days in order for sufficient and sustained knockdown of telomerase to occur. An acute AdR treatment at various concentrations (0.0-0.75μM) for 2 hours was done, followed by sample harvest at 2 and 4 days post-treatment. Growth of the cell lines was calculated to determine if pre-treatment with the siRNAs followed by AdR produced an effect (i.e. sensitized the cells) (Figure 16). As compared to the control MCF-7, cells transfected with the hTERT-1 siRNA displayed signficantly slowed growth by day 2 at all concentrations of AdR administered and this continued with the day 4 sample. As for those cells transfected with the hTERT-2 siRNA, considerably reduced levels of growth were observed at every concentration of AdR except 0.75μM. Finally, for those cells treated with the Pooled siRNA, differences in growth when compared to MCF-7 cells occurred on day 2 only at concentrations of 0.08 and 0.25μM AdR, but by day 4, sensitization of cells was seen at concentrations as low as 0.02μM AdR as well as 0.08 and 0.25μM AdR

We also measured cellular senescence occurred within the cells post-treatment in order to determine if senescence could be induced regardless of telomerase activity or telomere length. We found high levels of senescence in the cells transfected with hTERT-1 siRNA for both days and at every concentration of treatment (Figure 16). The hTERT-2 transfected cell lines only showed significant levels of senscence at day 2 with AdR concentrations of 0.25 and 0.75μM as well as at day 4 at 0.25μM. Similarly, the MCF-7 cells containing Pooled siRNAs revealed elevated levels of senescence at day 2 with AdR concentrations of 0.25 and 0.75μM but by day 4 cells treated with 0.08μM AdR also showed significant levels of senescence.

Sensitization of the cells was also tested by measuring levels of apoptosis (Figure 17). Cells transfected with any of the three sets of siRNA showed elevated levels of cell death by day 2 and continued on day 4 with all concentrations tested 0.0-0.75μM AdR with the largest percent apoptosis found in the hTERT-1 siRNA cell line followed by hTERT-2 and Pooled siRNA cell lines. Thus, regardless of the siRNA or combination of siRNAs utilized to inhibit telomerase, higher levels of cell death as compared with the MCF-7 cell controls were observed.

In order to compare sensitization of MCF-7 breast tumor cells to different types of chemotherapeutic drugs, we again utilized three sets of siRNAs (hTERT-1, hTERT-2, and Pooled) as a pre-treatment to exposure to taxol. The same experimental method was utilized as with AdR. Briefly, the siRNAs (150pmol) were transfected into the cells, and 3 days later, the cells were administered a 2 hour acute taxol treatment at various concentrations. Growth of the cell lines was calculated to investigate the effect pre-treatment with the siRNAs had on taxol treated MCF-7 cells (Figure 18). The first time

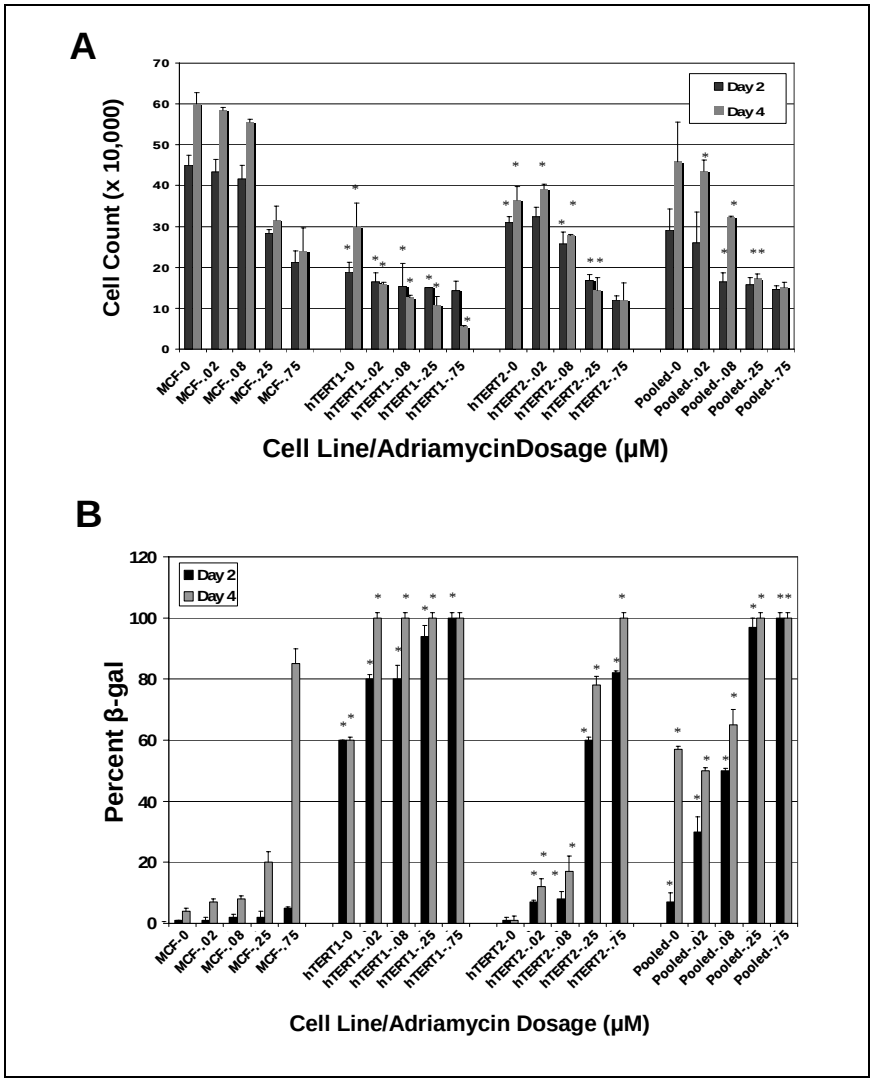

Figure 16. Decreased Growth and Increased Senescence in MCF-7 Cells Post-Treatment with Synthetic siRNAs and Adriamycin (AdR). Cells were transfected with the siRNA constructs (150pmol). Three days later, cells were treated with various concentrations of AdR. Samples were then taken two and four days post-treatment. **A.** Cell numbers were counted in duplicate. In the hTERT1 and hTERT2 transfected cell lines growth had slowed significantly, as compared with controls, at all dosages tested with hTERT-1 siRNA cell line revealing the most severe reduction of growth**. B.** The hTERT-1 transfected cells displayed the greatest percentage of senescent cells on both days and at every drug concentration. While not as successful, the Pooled siRNA cell line did show significant levels of senescence on day 2 at concentrations as small as 0.08µM AdR. Columns represent the mean from 3 representative fields of 100 cells; bars, SD; *, $P < 0.05$.

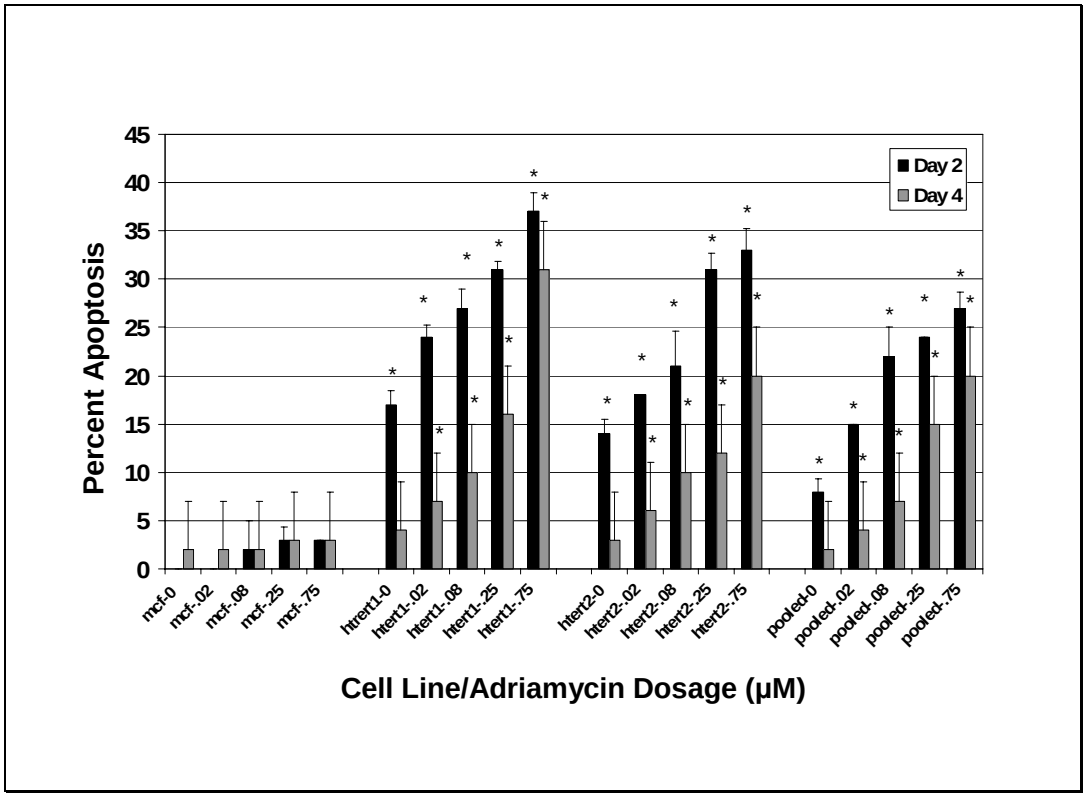

Figure 17. Apoptosis in MCF-7 Cells Post-Treatment with Synthetic siRNAs and Adriamycin (AdR). Same experimental procedure as Figure 30 except that the cells are stained for TUNEL. All cell lines transfected with three sets of siRNA revealed significantly high levels of apoptosis after AdR treatment with the largest percent apoptosis found in the hTERT1 siRNA cell line followed by hTERT2 and Pooled siRNA cell lines. Columns represent the calculated mean from 3 representative fields of 100 cells; bars, SD; *, $P < 0.05$.

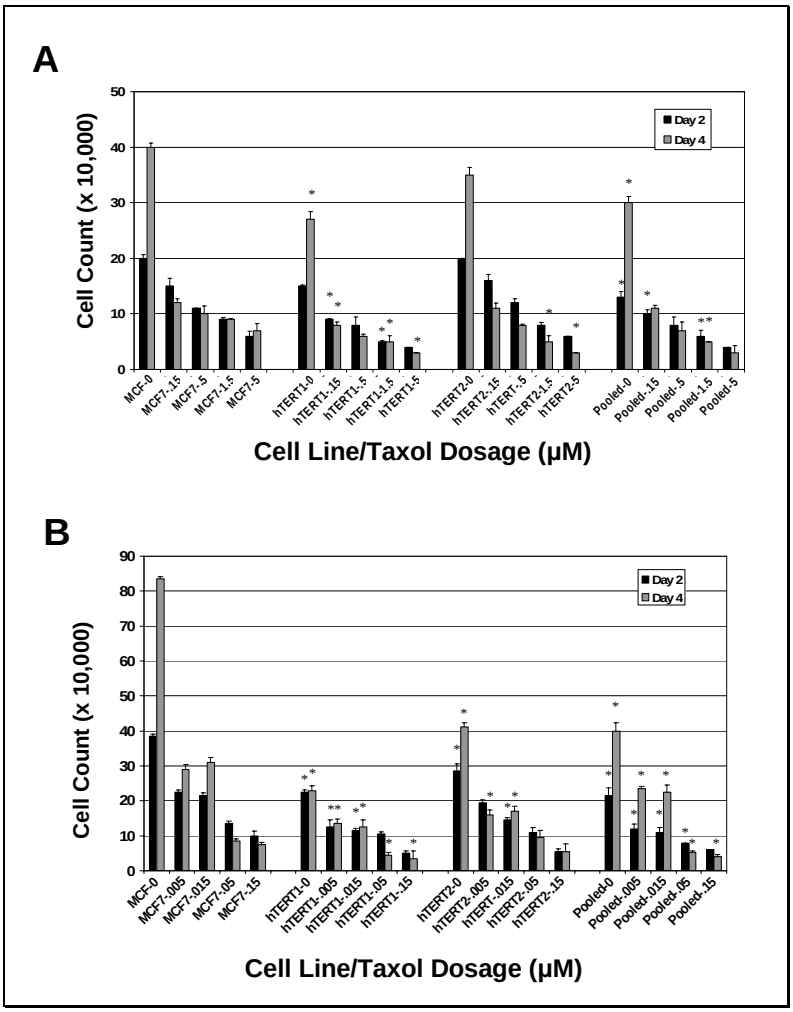

Figure 18. Growth of MCF-7 Cells Post-Treatment with Synthetic siRNAs and Taxol. Cells were transfected with siRNA constructs as indicated. Three days later, cells were treated with various concentrations of taxol. Samples were then taken 2 and 4 days post-treatment and counted in duplicate. **A.** With the higher concentrations, the taxol concentrations were too toxic for both the control and siRNA cell lines. **B.** Significantly slowed growth was observed all siRNA transfected cell lines after treatment with the lower concentrations of taxol. Significance for both graphs was determined by a two-tailed t-test and displayed on the graphs. *, $P < 0.05$.

the experiment was conducted the range of taxol (0.0-1.5μM) was too toxic, as before with the hTR siRNA experiments (see Figure 9). Therefore, the experiment was repeated with a lower scale of taxol dosages (0.0-0.15μM). As compared to the control MCF-7, cells transfected with the hTERT-1 siRNA displayed signficantly slowed growth by day 2 in the range of 0.0-0.015μM taxol, but by day 4 all samples showed significantly slower growth. As for those cells transfected with the hTERT-2 siRNA, considerably reduced levels of growth were observed by day 4 in the range of 0.0-0.015μM taxol and on day 2, growth was considerably slower for the cells treated with taxol at 0.0 and 0.015μM. Finally, for those cells treated with the Pooled siRNA, differences in growth when compared to MCF-7 cells occurred on both days at all concentrations except for day 2 at concentrations of 0.15μM taxol.

We also observed cellular senescence occurred within the cells post-treatment in order to determine if senescence could be induced regardless of telomerase activity or telomere length. We found high levels of senescence in the cells transfected with hTERT-1 for both days and at every concentration of treatment (Figure 19). The hTERT-2 transfected cell lines only showed significant levels of senscence at day 2 with taxol concentrations of 0.0, 0.005, 0.015 and 0.15μM as well as at day 4 at 0.015-0.15μM. Similarly, the MCF-7 cells containing Pooled siRNA revealed elevated levels of senescence at day 2 with taxol concentrations of 0.005 and 0.015μM but by day 4 at cells treated with 0.0 and 0.005μM taxol also showed significant levels of senescence.

Sensitization of the cells was also tested by measuring levels of apoptosis (Figure 19). Cells transfected with hTERT-1 and Pooled siRNAs showed elevated levels of cell death with all concentrations as well as for both samples. As for cell lines infected with hTERT-2, at day 2 cell lines treated with concentrations of 0.015 and 0.15μM showed significantly high levels of apoptosis but interestingly on day 4 only those cells administered dosages of 0.005 and 0.05μM taxol showed appreciable percent cell death.

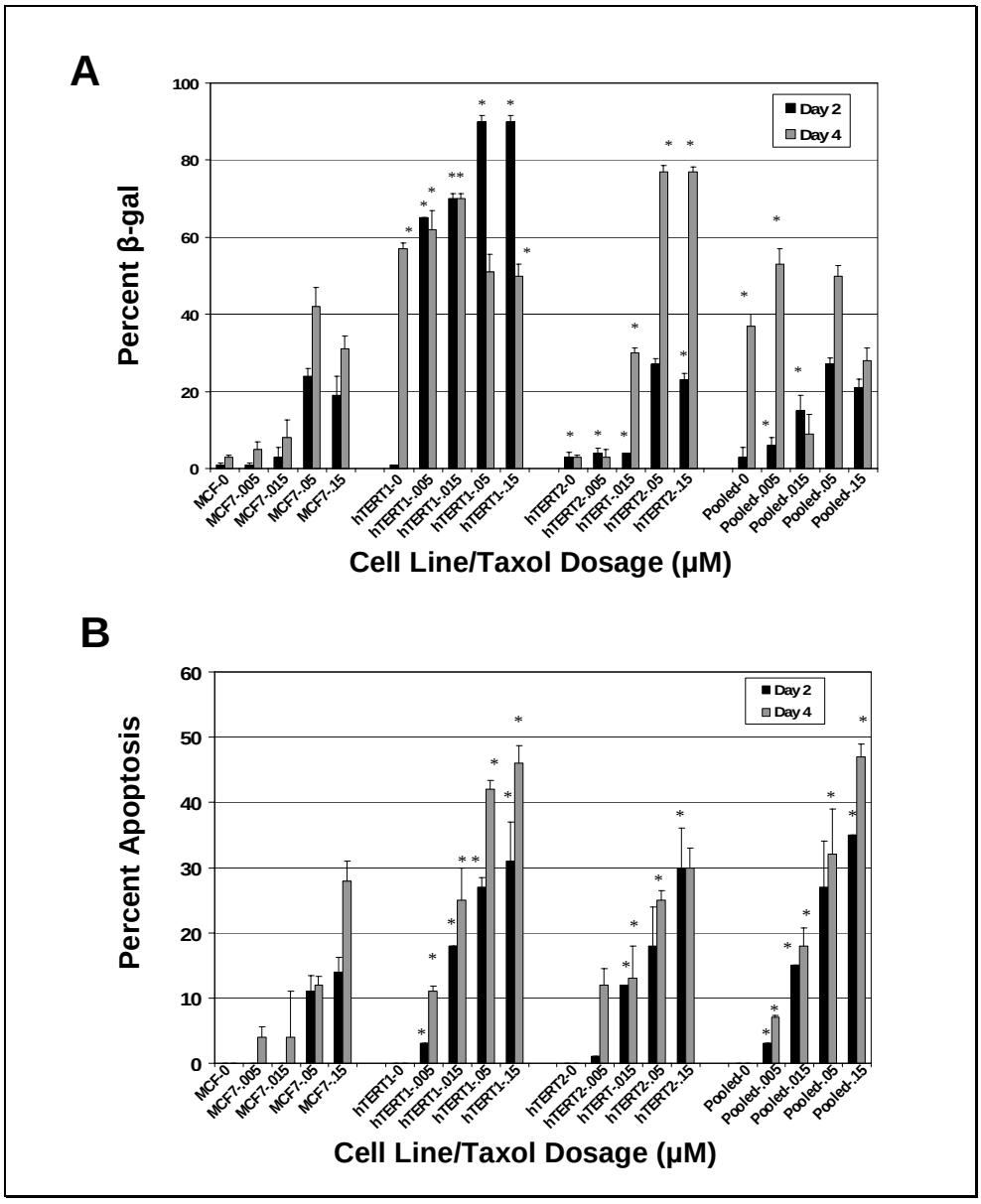

Figure 19. Cells Treated with Synthetic siRNAs and Taxol Showed Increased Sensitivity. Experimental methods are the same as in Figure 33. Cells were in fixed and incubated overnight with a β-gal staining solution. **A.** In the hTERT1 and hTERT2 siRNA transfected cell lines, noteworthy differences in senescence were observed at all taxol concentrations on both days samples were taken after taxol treatment**. B.** TUNEL assay showing percent apoptosis with hTERT1 revealing the greatest induction of cell death at all concentrations of taxol tested**.** Columns are the mean from 3 representative fields of 100 cells; bars, SD; *, $P < 0.05$.

Objective #5: Determine sensitization of DN-hTERT clones to AdR

The rate-limiting component of the telomerase complex is hTERT expression levels. Somatic cells contain only hTR and are without telomerase activity, whereas the majority of tumorigenic cells have telomerase activity because they express hTERT. Therefore, we looked at the effects of telomerase inhibition using DN-hTERT in breast tumor cells (MCF-7). We have shown previously that infection with DN-hTERT causes decreases in telomerase activity, hTERT RNA levels, and telomere lengths. We also showed high levels of senescence among the clonal populations that correlated with the level of DN-hTERT transgene expression.

Previous studies have established increased sensitivity to chemotherapeutic drugs after telomerase inhibition in a variety of tumor cell lines including lung cancer, cervical cancer and leukemia (Misawa et al. 2002; Nakamura et al. 2005; Ward and Autexier, 2005). Therefore, we wanted to assess if expression of DN-hTERT causes increased sensitization in breast cancer cells, specifically MCF-7 cells. Using colony forming assays, we examined the cells' ability to survive and proliferate after treatment with the DNA-damaging agent AdR. Three of the DN-hTERT clones with differing amounts of telomerase activity and telomere lengths were selected roughly 2 months post-infection for sensitization by AdR and tested for colony forming efficiency. Approximately, 2000 cells were seeded in triplicate and were administered an acute AdR treatment for 2 hours with varying concentrations (0.0-0.75μM). Ten days later, plates were stained with crystal violet and colonies counted. In comparison to the MCF-7 and pBABE cell lines, all of the DN-hTERT clones exhibited a greater sensitivity to AdR as seen by the considerable reduction in the number of colonies (Figure 20). Clonal growth rates were ascertained as critically lower than controls in all of the concentrations except at 0.0625μM in the DN9 cell line, which are significantly smaller than clinically relevant dosages at 0.75μM. Furthermore, clone DN9, which has longer telomeres and less DN-hTERT expression than the other clones (hence, only slightly inhibited telomerase activity), showed sensitization as low as 0.125μM AdR.

This increased sensitivity of the clonal populations with shorter telomeres implies that there is increased susceptibility of shortened telomeres to AdR and thereby DNA damage, which will be significant in the induction of apoptosis or senescence. Therefore, we wanted to determine protein levels of p53 and p21 post-treatment with AdR. Since we saw higher levels of senescence in clones with elevated telomerase inhibition, we expect to see induction of p53 because it is required for senescence to occur. We utilized the same clones (DN2, DN6 and DN9) and administered a 2 hour acute AdR treatment with varying concentrations (0.0-0.75μM) (Figure 21). We assessed up-regulation of the DNA damage repair proteins at 6 and 24 hours after AdR treatment.

Basal levels of p53 (i.e. untreated cells) were elevated in all DN-hTERT clones with especially high quantities in the DN2 clone. However, p21 was only up-regulated in clones DN2 and DN6, implying a constant level of stress within the clonal populations. After exposure to AdR, the only difference, as compared to the control pBABE cells, was at the 6 hour time point at 0.75μM with up-regulation of p53 and significantly higher levels of p21. Also observed at 6 hours was the induction of high levels of p21 in clones DN2 and DN6 after treatment with 0.25μM AdR.

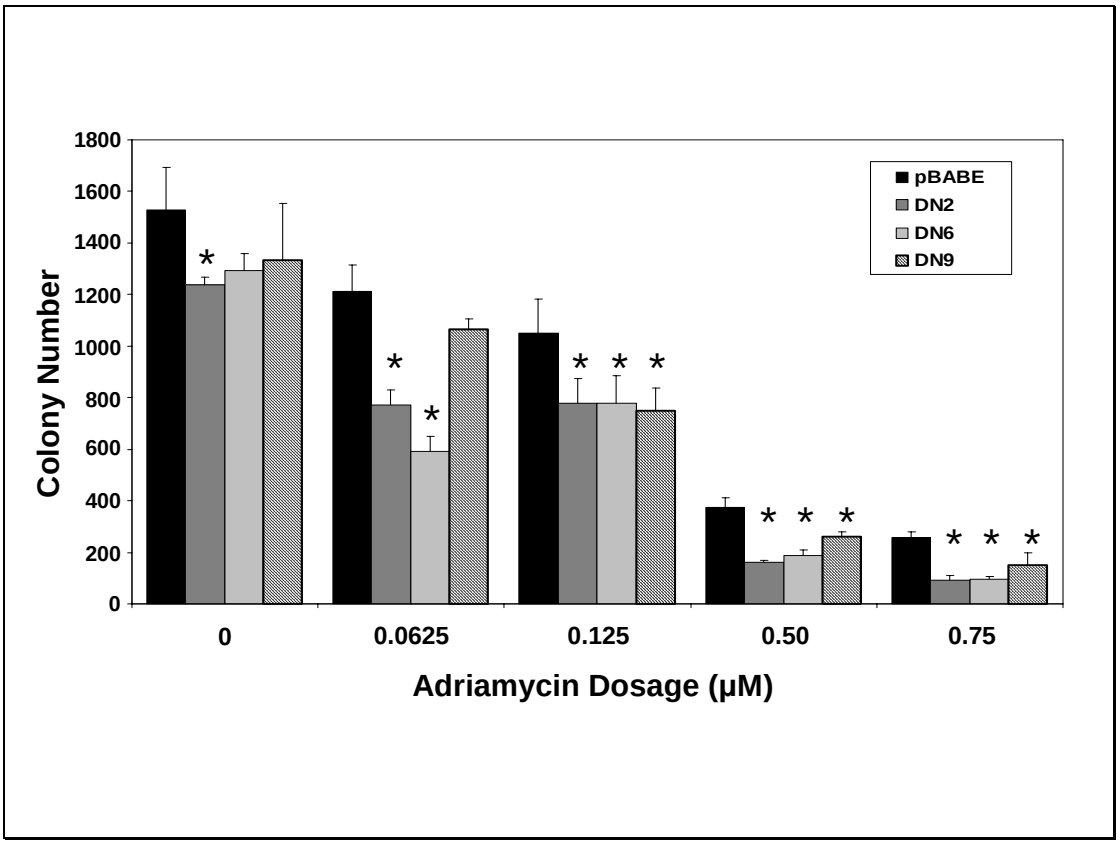

Figure 20. Increased Sensitivity after AdR Treatment. DN-hTERT/ MCF-7 cells were treated with a 2 hour acute treatment with various amounts of AdR. Colonies were stained and counted after 10 days of growth. The three DN-hTERT clones displayed significantly increased sensitivity as seen by decreased colony growth after treatment at lower dosages. Clonal differences in sensitivity correlated to levels of DN-hTERT expression and telomerase activity within the population.

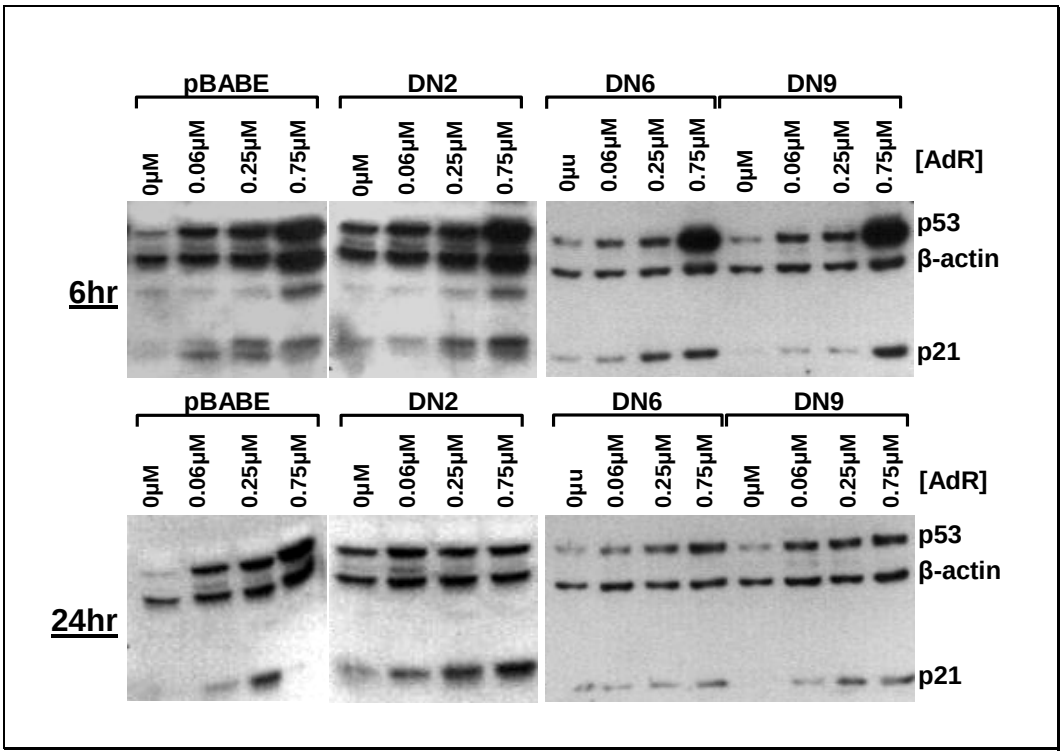

Figure 21. Induction of DNA Damaging Proteins after Treatment with AdR. DN-hTERT/ MCF-7 cells were treated with a 2 hour acute treatment with various amounts of AdR. Samples were taken 6 and 24 hours post-treatment. Cell pellets were collected and 15μg of protein lysate was subject to Western Blot analysis. Immunoblots were probed with anti-p53, anti-p21, and anti-actin as a loading control. The basal levels of p53 in all DN-hTERT clones were elevated as compared to controls with especially high levels found in the DN2 cell line.

Objective #6: Determine the long term effects of the DN-hTERT in breast tumor cells

Instead of the projected lag time of growth and telomere shortening followed by death or senescence, we observed from these long-term cultures the emergence of surviving cells that reverted back to normal phenotype and growth rate. Furthermore, these cells regained higher levels of telomerase activity than those found in normal MCF-7 and pBABE control cell lines (Figure 22).

Previous studies have also shown the reactivation of telomerase in human leukemia cell lines (AML and PML) and a murine kidney tumor cell line after telomerase inhibition with a dominant negative TERT (Delhommeau et al. 2002; Klapper et al. 2003; Sachsinger et al. 2002). To uncover the reason for this recovery, we examined the RNA levels of endogenous and exogenous hTERT using RT-PCR (Figure 23). We wanted to compare expression levels before and after recovery as well as detect differences in RNA levels between the clones. Similar levels of exogenous DN-hTERT were detected as before when telomerase activity was decreased. However, clonal variability was not found after recovery, and high levels of endogenous hTERT and DN-hTERT were observed (Figure 43). Therefore, after the clones had recovered, the levels of telomerase activity did not correlate with RNA expression levels of DN-hTERT, possibly suggesting a mechanism independent of transcriptional upregulation of hTERT.

Consequently, we wanted ascertain how the hTERT protein levels were affected within the clones by the reactivation of telomerase activity using a western blot analysis (Figure 23). We looked at protein expression in five of the DN-hTERT clones and found considerably higher levels of hTERT compared to those seen previously, not to mention the fact that hTERT levels appeared equivalent in intensity as compared to the pBABE/MCF-7 control cell line. Thus, even in the presence of the same levels of DN-hTERT RNA, endogenous hTERT protein was maintained at a constant level, essentially equivalent to that of the MCF-7 cells, indicating that the DN-hTERT is being inactivated or overtaken by the higher levels of hTERT protein.

With this recovery or reactivation of telomerase activity we wanted to ascertain if this also resulted in elongation of the telomeres. Four clones were analyzed approximately three to four months post-infection with DN-hTERT using the TALA assay (Figure 24). All of the clones appeared to have undergone a slight lengthening in overall telomere size, but telomere elongation was especially apparent in clones DN2 and DN10. Recent studies have demonstrated that telomerase will elongate the telomeres that are most likely to be involved in chromosomal abnormalities that cause genomic instability (der-Sarkissian et al. 2004; Zou et al. 2004), so even though the average size of the telomeres were not vastly different when compared to earlier clones during crisis, this could be due to the preferential elongation of the shortest telomeres within the cell and perhaps overtime the other telomeres will continue to lengthen.

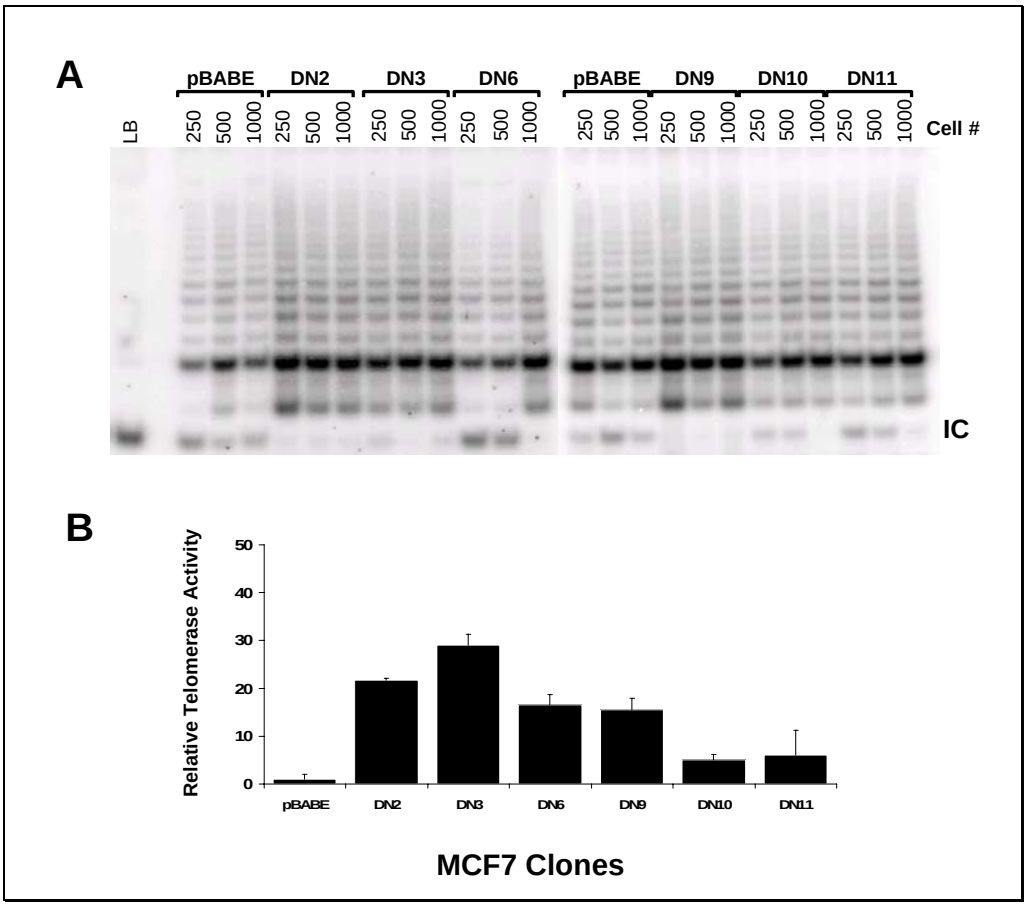

Figure 22. Loss of Telomerase Repression and Recovery. Approximately 4 months post-infection, the DN-hTERT cells went through "crisis" and telomerase activity was detected as a measure of recovery. **A.** Representative TRAP assay showing 250, 500, and 1000 cell equivalents of the DN-hTERT/MCF7 breast cancer cells. **B.** Quantitation of the relative telomerase activity was accomplished as stated in Figure 36. Telomerase activity was found to be highly up-regulated in all clones with a minimum of a 5 fold increase than in the pBABE/MCF-7 controls.

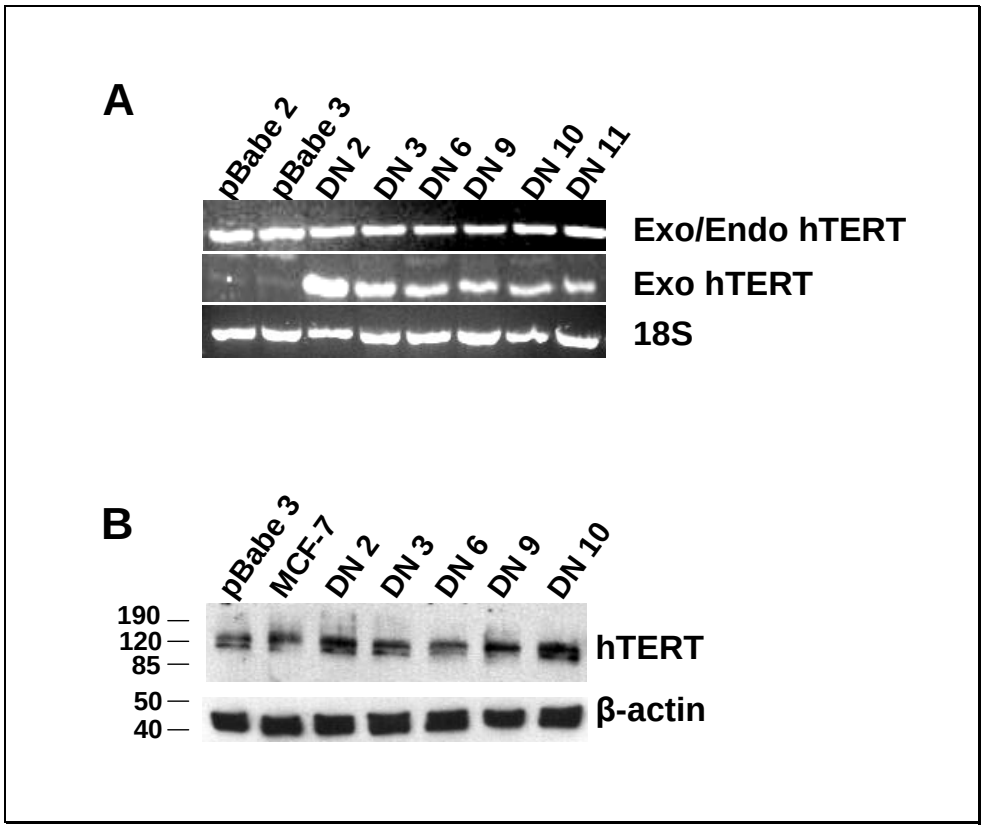

Figure 23. hTERT Expression Levels are Elevated Post-Recovery. A. RT-PCR was conducted to determine the expression of both the overall levels of hTERT (exo/endo) as well as the exogenous DN-hTERT (exo) levels in the various clonal populations. **B.** Cell pellets were collected and 20μg of protein lysate was subject to Western analysis. Immunoblots were probed with anti-hTERT with anti-actin serving as a loading control. Similar high levels of exogenous DN-hTERT were detected as 3 months previously when telomerase activity was knocked-down.

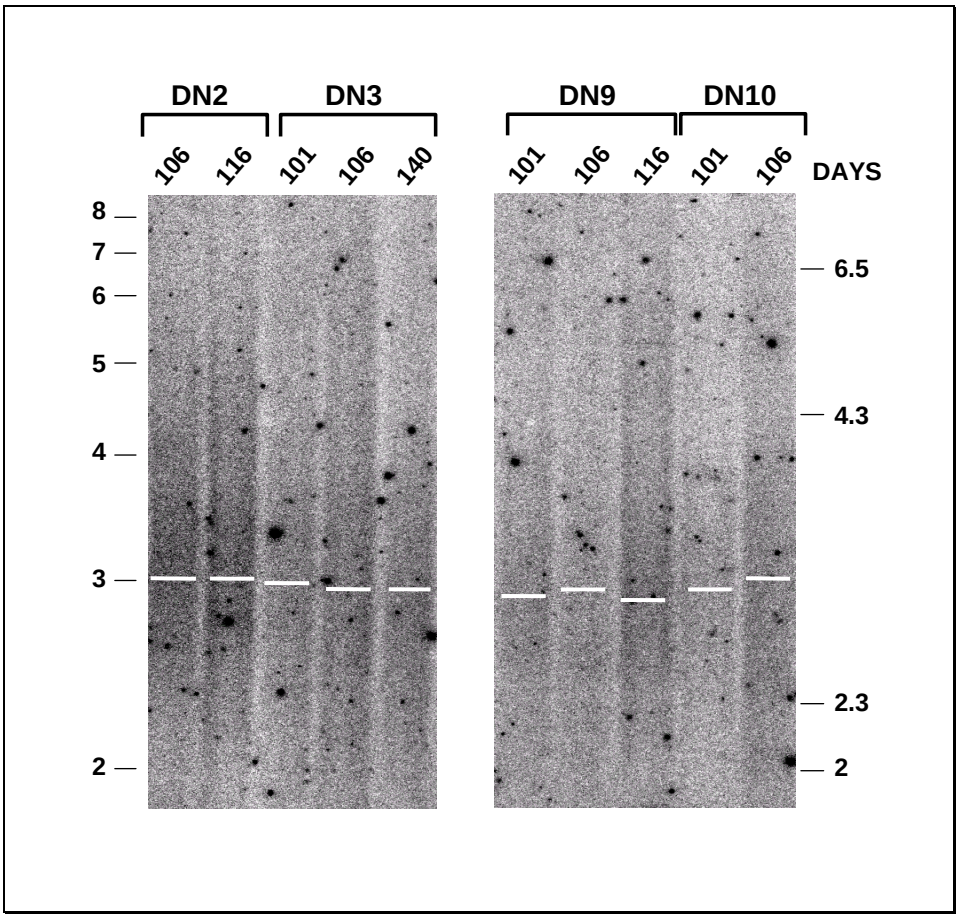

Figure 24. Telomere Lengths were Assessed Post-Recovery. Samples were sequentially collected between month 3 and 4 in four different clonal populations and telomere lengths were determined by the TALA assay. DN-hTERT/MCF-7 clones 2 and 10 exhibited elongated telomeres as time progressed and as the cell lines reactivated telomerase.

Objective #7: Comparison of Senitization of Clone DN2 During Crisis and Post-Recovery

First, we tested if DN-hTERT expression resulted in sensitization of clonal populations to AdR during crisis and after re-activation of telomerase post-recovery. Crisis is defined by the characterizations of the cell line observed earlier including telomerase inhibition, shortened telomeres, slowed growth, decreased levels of hTERT and a senescent-like state. Cells were exposed to an acute 2 hour treatment with various concentrations of AdR (0.0-0.75μM). Samples were taken 2 and 4 days post- treatment. Initially, we measured growth of the cell lines, in duplicate, in order to determine if the
chemotherapeutic agent slowed the growth of cells regardless of telomerase activity (Figure 25). In comparison with the pBABE control cell line, only the DN2 clones in crisis (i.e. with inhibited telomerase activity) showed significantly less growth for both days and every concentration. The AdR treatments appeared to have no effect on the recovered DN2 clonal cell line, which had almost equivalent growth as the pBABE cell line. Perhaps, the reactivation of telomerase provided a protective function for the telomeres because the most critically short telomeres had been elongated. Following growth, we assessed the induction of senescence with β-galactosidase expression. We attained the same results as with growth. Only the DN2 cells in crisis displayed significantly higher levels of senescence for every concentration and for both days (Figure 25). Other studies have shown induction of senescence in MCF-7 cells 4 days after exposure to 1μM AdR (Elmore et al. 2002). Therefore, generation of such high levels of senescence 2 days post-treatment and at concentrations of AdR as low as 0.02μM is extremely significant revealing definite sensitization of DN-hTERT cells to AdR. Lastly, we examined the percent apoptotic cells after treatment with AdR using the TUNEL assay (Figure 26). Neither population of cells revealed considerably high levels of cell death at day 2 or day 4. In fact, there were overall low levels of apoptosis at every concentration regardless of the cell line. However, this is not totally unexpected because other studies using MCF-7 cells have shown that only senescence occurs post-treatment with AdR due to the presence of p53 because once p53 is knocked-out using E6, increased apoptosis occurs (Elmore et al. 2002).

In order to provide another means of sensitization in MCF-7 breast cancer cells, we utilized taxol, which has a distinctive effect compared to AdR as explained above. The same sensitization experiment was used as with AdR. Briefly, cells were exposed to various concentrations of AdR (0.0-0.75μM) for 2 hours with samples taken 2 and 4 days post-treatment. Again, we analyzed growth and found only the DN2 clones in crisis showed significantly slowed growth at every concentration as compared to controls (Figure 27). Treatment with taxol had minimal effects on the growth of DN2 post-recovery as compared to pBABE controls. We also assessed senescence post-treatment with taxol and found only the DN2 clones in crisis displayed considerably higher levels within the cell as compared to controls (Figure 27). Finally, using the TUNEL assay we looked apoptosis after treatment with taxol (Figure 28). Significantly higher levels of apoptosis were observed in the DN2 crisis cell line but not so in the DN2 cell line post-recovery, suggesting that the DN2 cells behave more like parental MCF-7 cells than those with decreased telomerase.

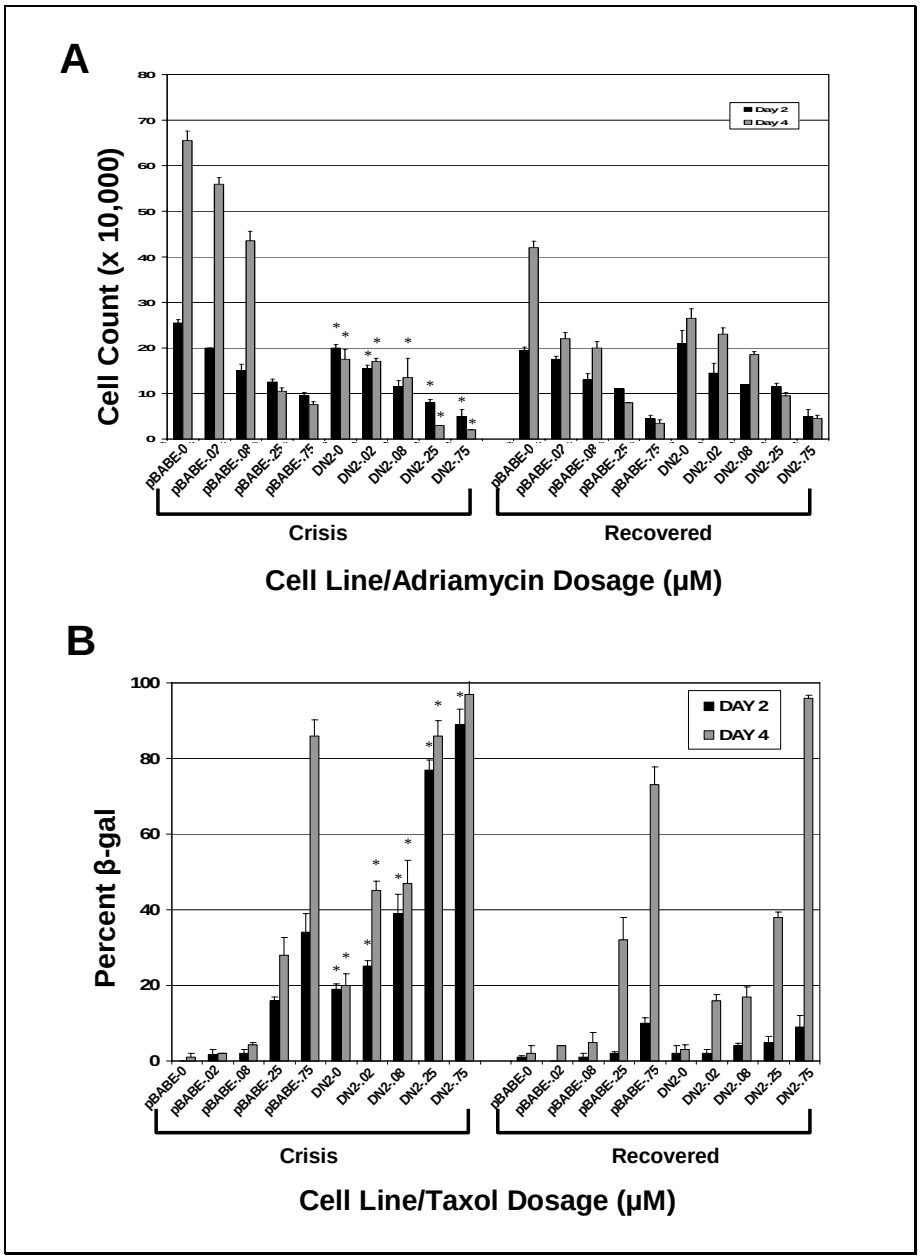

Figure 25. Decreased Growth and Increased Senescence in the DN2 Clone during Crisis but not in Recovered cells after Treatment Adriamycin (AdR). The DN2/MCF-7 clone at different stages, crisis and recovered, was exposed to various concentrations of AdR and samples were then taken 2 and 4 days post-treatment. **A.** Cell numbers were counted in duplicate. Only cells in crisis had significantly slower cellular growth compared to controls at dosages lower than clinically relevant. **B.** β-galactosidase staining for senescence, calculated for 3 independent fields of 100 cells. Similarly, only the DN2 cells in crisis displayed elevated levels of senescence at all concentrations of AdR. *, $P < 0.05$.

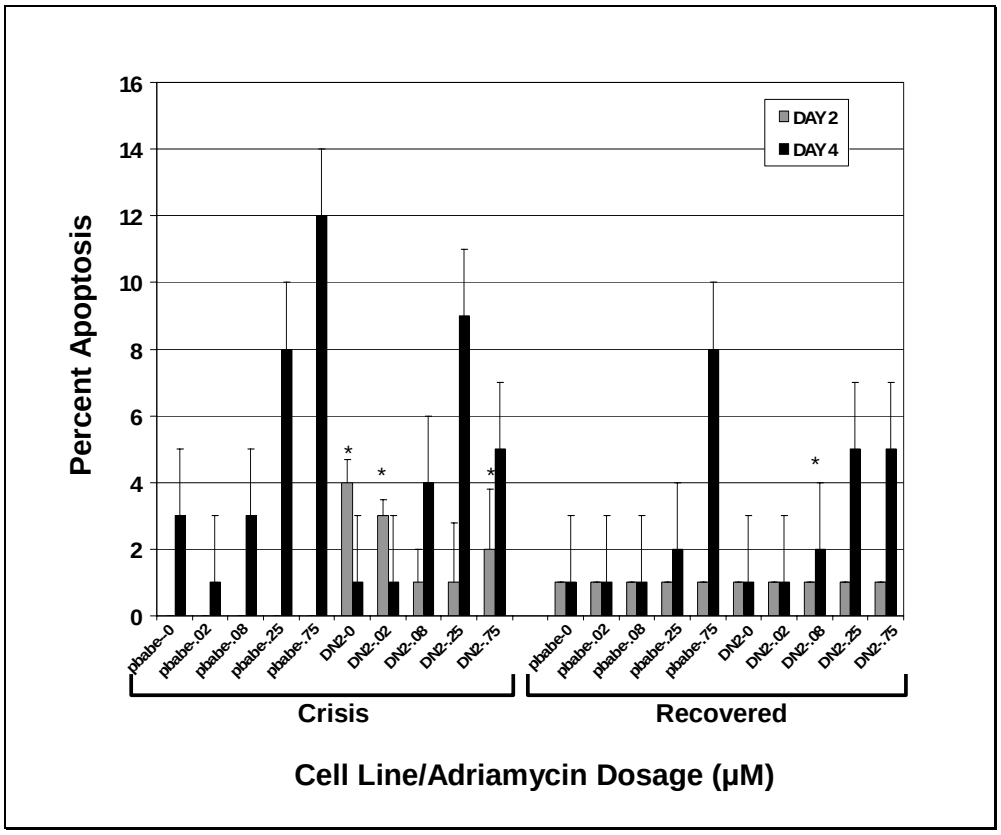

Figure 26. Sensitization of DN2 Cell Lines to Apoptosis after Treatment with AdR does not occur. Quantitation of a TUNEL assay 2 and 4 days post-treatment with AdR as was calculated in Figure 31. Neither the DN2 cells during crisis or after recovery revealed any substantial divergence in percent apoptosis as compared with the pBABE controls. *, $P < 0.05$.

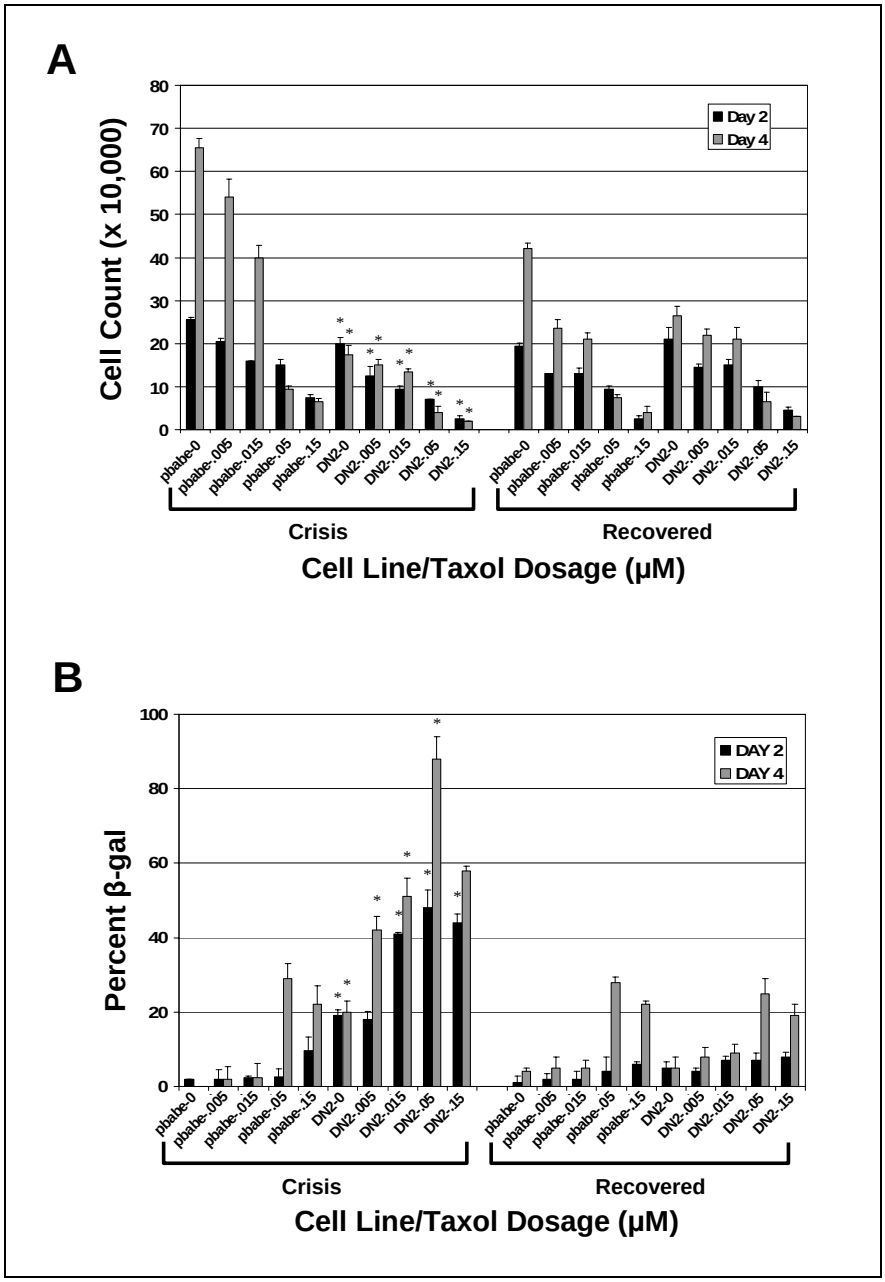

Figure 27. Taxol Causes Decreased Growth and Increased Senescence in the DN2 Clone during Crisis but not after Recovery. The DN2/MCF-7 clone at different stages, crisis and recovered, was exposed to various concentrations of taxol and samples were then taken 2 and 4 days post-treatment. **A.** Cell numbers were counted in duplicate. Only cells in crisis had significantly slower cellular growth, as compared with controls, at dosages lower than clinically relevant. **B.** β-galactosidase staining was done and calculated for 3 independent fields of 100 cells. Similarly, only the DN2 cells in crisis displayed elevated levels of senescence at almost all concentrations of taxol. *, $P < 0.05$

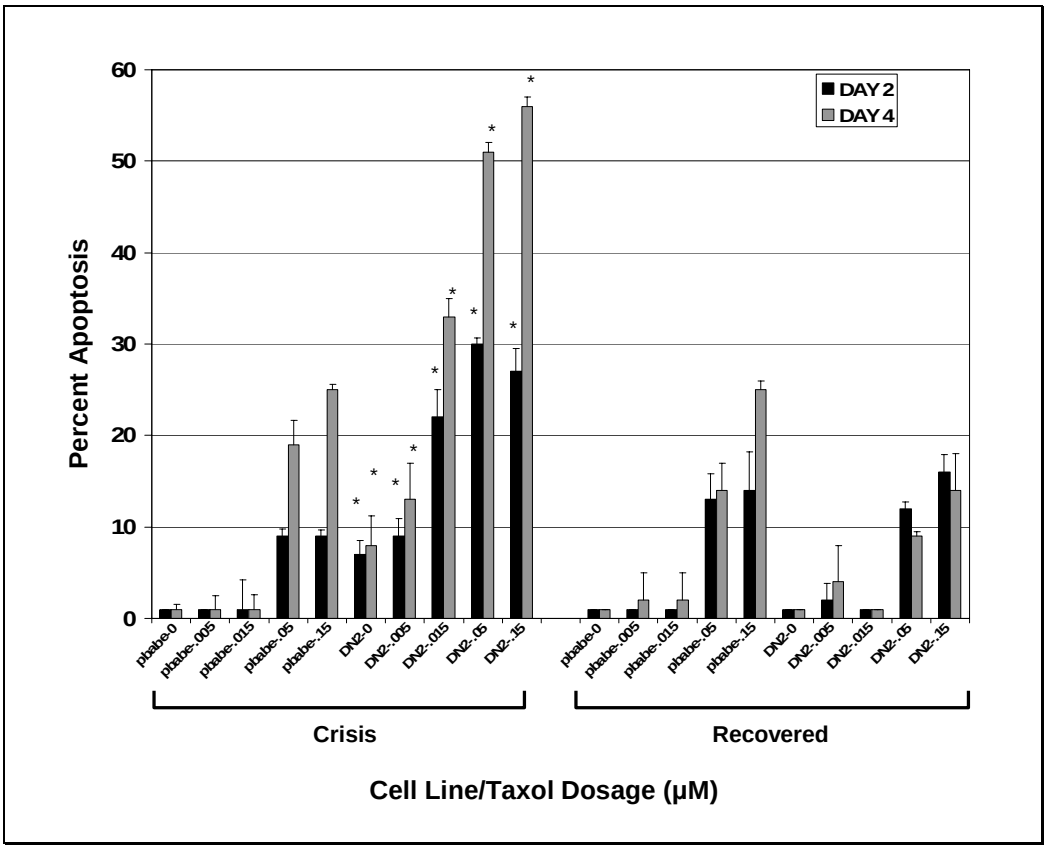

Figure 28. Sensitization of DN2 Cell Lines to Apoptosis after Treatment with Taxol. Quantitation of a TUNEL assay 2 and 4 days post-treatment with AdR. Only the DN2 cells during crisis revealed any substantial divergence in percent apoptosis as compared with the pBABE controls. *, $P < 0.05$.

Key Research Accomplishments

1-Transient inhibition of telomerase activity was observed using RNAi targeting hTR and hTERT. The two most effective siRNAs were hTR-2 and hTERT-1.

2-After transfection with the synthetic siRNAs we found significantly increased sensitization to the chemotherapies AdR and taxol at much lower concentrations than clinically relevant. Sensitization was measured by slowed growth in addition to increases in the levels of senescence and apoptosis.

3-DN-hTERT caused increased susceptibility of the telomeres to AdR resulting in significant differences in colony growth after acute treatment as well as induction of the DNA damage response proteins p53 and p21.

4-The unexpected finding of recovery of telomerase in the DN-hTERT cell lines after a certain amount of time indicating a window of opportunity for treatment. This was quantified by increases in telomerase activity, hTERT RNA levels, and telomere lengths.

5-Increased sensitization to standard chemotherapies of DN-hTERT clones in crisis as compared to the same clonal cell line post-recovery of telomerase activity.

Recommended Changes to the Proposed Work Based on Additional Findings

No additional changes to the proposal are requested.

Reportable Outcomes

Abstracts/Presentations

Poynter,K.R. Department of Defense: Era of Hope, Philadelphia, PA. June 2005

Poynter,K.R. VCU Institute for Women's Health: 2nd Annual Women's Health Research Day. Richmond, VA. March 2006.

Poynter,K.R. VCU: Massey Cancer Center Retreat, Richmond, VA. June 2006.

Poynter,K.R. VCU: Daniel T. Watts Research Poster Symposium, Richmond, VA. October 2006.

Publications

Poynter, K.R., Elmore, L.W., and Holt, S.E. 2006. Telomeres and telomerase in aging and cancer: Lessons learned from experimental model systems, Drug Discovery Today: Disease Models **3**:155-160.

Poynter, K.R., Elmore, L.W., and Holt, S.E. 2006. Sensitization of breast tumor cells using varying methods for telomerase inhibition. Manuscripts in Preparation.

Development of Cell Lines

We have developed cell lines for telomerase knockdown with synthetic siRNA targeting hTR and hTERT as well as both simultaneously. We have also blocked telomerase activity using a dominant-negative approach targeting hTERT (DN-hTERT). In addition we created two cell lines with a double knockdown via RNAi of p21 in MCF-7 cells. Other siRNA vectors that have been constructed and expressed in breast tumor cells but not completely characterized include several MCF-7 cell lines that have targeted knockdown of hTERT, using RNA interference.

Funding Applied For

Department of Defense Breast Cancer Research Program, Pre-doctoral award, May 2003-awarded.

Conclusions

Telomerase, a ribonucleoprotein enzyme minimally composed of an RNA template (hTR) and a catalytically active protein subunit (hTERT), synthesizes telomeric repeat**s** onto chromosome

ends and is obligatory for continuous tumor cell proliferation, as well as malignant progression of breast cancer cells. Telomerase is an attractive anti-cancer therapeutic target because its activity is present in over 90% of human cancers, including more than 95% of breast carcinomas, but undetectable in most somatic cells. Traditional chemo- and radio-therapies lack the ability to effectively control and cure breast cancer, in part because residual cells are or become resistant to DNA damaging modalities.

While various telomerase inhibition strategies cause cancer cells to undergo apoptosis or senescence, there is often a lag period between administration and biologic effect (Corey, 2002). Our goal in this study was to compare the efficacy of different telomerase inhibition strategies in concert with standard chemotherapeutic agents at triggering senescence and/or apoptosis in cultures of breast cancer cells. We hypothesized that telomerase inhibition strategies will sensitize breast cancer cells to traditional chemotherapies, potentially reducing the lag phase**,** allowing for more potent anti-tumor effects at lower doses, and therefore ultimately imparting less toxicity to the patient.

We blocked telomerase by targeting hTR and hTERT, individually and collectively utilizing synthetic short interfering RNA (siRNA), short hairpin RNA (shRNA) and a dominant negative form of hTERT (DN-hTERT) in MCF-7 breast cancer cells. We analyzed the efficiency of telomerase inhibition for each strategy alone and then treated the cells with two mainstay chemotherapeutic agents, Adriamycin (AdR) and Taxol. The most effective telomerase inhibition strategies were synthetic siRNA and DN-hTERT, individually. After treatment with various concentrations of AdR or Taxol, breast cancer cells with inhibited telomerase grew significantly slower and exhibited widespread senescence or apoptosis within a much shorter time period and at a dose that is insufficient to trigger cytostasis. In addition, we provide evidence that cells in which telomerase was inhibited were more sensitive to anti-cancer agents, whether the drug inhibited topoisomerase II resulting in DNA damage (AdR) or blocked mitosis via protracted microtubule stabilization (Taxol). Collectively, our data indicate that alone, anti-telomerase inhibition strategies differ in their efficacy. However, when used in the adjuvant setting with diverse acting chemotherapeutic agents, there is a potent synergy resulting in chemotherapeutic sensitization characterized in part by widespread senescence and/or apoptosis.

Abbreviations

hTERT-human telomerase reverse transcriptase; **hTR**-human telomerase template RNA; **AdR**-adriamycin; **siRNA**-short interfering RNA; **RNAi**-RNA interference; **SKY**-spectral karyotyping; **FISH**-fluorescent *in situ* hybridization; **SA-β gal**-senescence associated β -galactosidase; **ChIP**-chromatin immunoprecipitation; **TRAP**- telomere repeat amplification protocol (telomerase activity assay); **TRF**- terminal repeat fragment (telomere length assay); **TRF2**- telomere repeat binding factor number 2; **IP**- immunoprecipitation; **TnT**- transcription and translation.

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